สารยับยั้งแอลฟากลูโคซิเดสจากใบมะรุม *Moringa oleifera* และกากเมล็ดงา *Sesamum indicum*

นางสาวอริน วิกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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α-GLUCOSIDASE INHIBITORS FROM *Moringa oleifera* LEAVES AND *Sesamum indicum* SEED PULPS

Miss Arin Wikul

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Field of Study Biotechnology

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อริน วิกุล : สารยับยั้งแอลฟากลูโคซิเดสจากใบมะรุม *Moringa oleifera* และกากเมล็ดงา *Sesamum indicum* (α-GLUCOSIDASE INHIBITORS FROM *Moringa oleifera* LEAVES AND *Sesamum indicum* SEED PULPS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ปรีชา ภูวไพรศิริศาล, 54 หน้า

การศึกษาสารยับยั้ง α-glucosidase เป็นแนวทางหนึ่งที่มีประสิทธิผลในการรักษาโรคเบาหวานชนิดที่ 2 ได้เป็นอย่างดี จึงพยายามศึกษาสารยับยั้ง α-glucosidase จากพืชสมุนไพรไทย ซึ่งในงานวิจัยนี้ ได้ทำการศึกษาพืชสมุนไพรไทย 2 ชนิดคือ ใบมะรุมและกากเมล็ดงา ซึ่งเป็นพืชกินได้และมีแพร่หลายในประเทศไทย โดยอาศัยวิธีทางโครมาโทรกราฟีและทดสอบฤทธิ์ทางชีวภาพนำไปสู่การแยกสารบริสุทธิ์ที่มีฤทธิ์ยับยั้ง α-glucosidase การแยกส่วนสกัดเมทานอลจากใบมะรุมได้สารฟีนอลิคไกลโคลไซด์ 2 ชนิดคือ niazirin และ pyrrolemarumine 4"-*O*-α-L-rhamnopyranoside และสารฟลาโวนอยด์ไกลโคลไซด์อีก 2 ชนิดคือ kaempferol-3-*O*-*β*-glucopyranoside และ quercetin-3-*O*-*β*-glucopyranoside นำสารที่แยกได้มาทดสอบฤทธิ์ยับยั้ง α-glucosidase พบว่า มีเพียงสารฟีนอลิคไกลโคลไซด์มีฤทธิ์ยังยั้ง α-glucosidase จากเบเกอร์ยีสต์ โดย niazirin มีฤทธิ์ยับยั้ง α-glucosidase ได้มากกว่า acarbose ซึ่งเป็นยารักษาโรคเบาหวาน ถึง 8 เท่า ขณะที่ สารฟลาโวนอยด์ไกลโคลไซด์มีฤทธิ์ยังยั้ง α-glucosidase จากลำไส้หนู โดย quercetin-3-*O*-*β*-glucopyranoside มีฤทธิ์ยับยั้งเอนไซม์มอลเทส (IC_{50} 95.73 ไมโครโมล) และซูเครส (IC_{50} 54.80 ไมโครโมล) ในส่วนของกากเมล็ดงา พบว่าส่วนสกัดเมทานอลสามารถแยก สารลิกแนนได้ 3 ชนิดคือ sesamin, sesamolin และ pinoresinol นำสารที่แยกได้มาทดสอบฤทธิ์ยับยั้งเอนไซม์ α-glucosidase พบว่าลิกแนนทั้งสามชนิดออกฤทธิ์ยังยั้ง α-glucosidase จากเบเกอร์ยีสต์ (ช่วง IC_{50} เท่ากับ 0.20-0.49 มิลลิโมล) ขณะที่ pinoresinol มีฤทธิ์ยังยั้ง α-glucosidase จากลำไส้หนู โดยยับยั้งเอนไซม์มอลเทส (IC_{50} 0.0343 มิลลิโมล) การศึกษากลไกการยับยั้ง α-glucosidase โดยการวิเคราะห์ทางไคเนติกส์ของ pinoresinol พบว่า เป็นการยับยั้งแบบ mixed-type inhibition ซึ่งผลที่ได้ทางไคเนติกส์จากงานวิจัยนี้ ยังไม่มีการรายงานมาก่อน

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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ARIN WIKUL: α-GLUCOSIDASE INHIBITORS FROM *Moringa oleifera* LEAVES AND *Sesamum indicum* SEED PULPS. ADVISOR: ASST. PROF. PREECHA PHUWAPRAISAN, 54 pp.

α-Glucosidase inhibitors and applied as the effective therapeutic approach for treatment of type II diabetes. We have examined the inhibitory effect of Thai medicinal plants against α-glucosidase. The *Moringa oleifera* leaves and *Sesamum indicum* defatted seeds, which are edible plants and cultivated in Thailand, were selected for this investigation are Chromatography technique and bioassay-guided fractionation led to the isolation of active components. The isolation of methanol crude extract from *M.oleifera* leaves afforded two phenolic glycosides named niazirin and pyrrolemarumine 4"-*O*-α-L-rhamnopyranoside and two flavonoid glycosides named kaempferol-3-*O*-*β*-glucopyranoside and quercetin-3-*O*-*β*-glucopyranoside. All the isolated compounds were tested for inhibitory activity against α-glucosidase. Only phenolic glycosides selectively inhibited α-glucosidase from baker's yeast. Niazirin was the most potent α-glucosidase inhibitor against baker's yeast, which was approximately 8 times more active than that of antidiabetic drug acarbose whereas flavonoid glycosides selectively inhibited that from rat intestine; in which the most active compound quercetin-3-*O*-*β*-glucopyranoside inhibited maltase (IC_{50} 95.73 μM) and sucrase (IC_{50} 54.80 μM). On the other hands, the isolation of methanol crude extract from *S.indicum* defatted seeds afforded three lignans named sesamin, sesamolin and pinoresinol. They broadly inhibited α-glucosidase from baker's yeast (IC_{50} 0.20-0.49 mM), whereas pinoresinol selectively inhibited maltase from rat intestine (IC_{50} 0.0343 mM). To gain insight into inhibition mechanism of pinoresinol, kinetic study was carried out. A kinetic analysis showed that pinoresinol inhibited maltase by mixed-type manner. Notably, the inhibition mechanism of pinoresinol is first reported herein.

Field of Study : Biotechnology Student's Signature .....

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LIST OF ABBREVIATIONS

A	absorbance, 2′-deoxyadenosine (in a DNA sequence)
bp	base pairs
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
C	2′-deoxycytidine (in a DNA sequence)
^{o}C	degree Celsius
Da	Dalton
DAHDH	L-erythor-3,5-diaminohexanoate
DAPDH	2,4-diaminopeptanoate dehydrogenase
DAPMDH	meso-2,6-diaminopimelate dehydrogenase
DH	dehydrogenase
DNA	deoxyribonucleic acid
dNTP	2′-deoxynucleoside 5′-triphosphate
EC	Enzyme Commission
EDTA	ethylene diamine tetraacetic acid
G	2′-deoxyguanosine (in a DNA sequence)
HPLC	high-performance liquid chromatography
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
kb	kilobase pairs in duplex nucleic acid, kilobases in single-standed nucleic acid
KCl	potassium chloride
kDa	kiloDalton
K_m	Michaelis constant
KOH	potassium hydroxide
kV	kilovolt
l	liter
LB	Luria-Bertani
leudh	L-leucine dehydrogenase gene
*leu*E	leucine exporter gene

LeuDH	L-leucine dehydrogenase
µF	microfarad
µg	microgram
µl	microliter
µM	micromolar
M	mole per liter (molar)
mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MethylalaDH	N-methyl-L-alanine dehydrogenase
M_r	relative molecular mass
MW	molecular weight
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
$NaHCO_3$	sodium carbonate-bicarbonate buffer
Na_2HPO_4	disodium hydrogen orthophosphate
ng	nanogram
nm	nanometer
NH_4Cl	ammonium chloride
$(NH_4)_2SO_4$	ammonium sulfate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pI	isoelectric point
pmol	picomole
PMSF	phenyl methyl sulfonyl fluoride
RNase	ribonuclease
SDS	sodium dodecyl sulfat

T	2′-deoxythymidine (in a DNA sequence)
TB	Tris-borate buffer
TE	Tris-EDTA buffer
TEMED	*N, N, N′, N′*-tetramethyl ethylene diamine
TLC	thin-layer liquid chromatography
T_m	melting temperature, melting point
UV	ultraviolet
V	voltage
v/v	volume by volume
w/w	weight by weight

CHAPTER I

Introduction

Diabetes mellitus (DM), commonly referred to as diabetes, is a group of metabolic disease characterized by elevated blood glucose levels that result from detects pancreatic insulin secretion with or without concurrent impairment of insulin action. DM is increasingly being acknowledged that diabetes and other chronic are major public health problem. It is a condition that is increasing in epidemic states throughout the world. According to the World Health Organization (WHO, 2006), the worldwide evaluation of the disease around 2030 will be more than double from that of 2005, which account for an increase of 144% over the next 30 years (Gershell *et al*., 2005).

Insulin is a hormone, which is released from the pancreas and controls the amount of glucose in the blood boarder. When human eats or drinks, food is digested into glucose. Glucose is absorbed into the bloodstream and stimulates pancreas to produce insulin. Therefore, Glucose is transported into cell when insulin bind to insulin receptor, which straddles cell membrane of many cell (Fig 1.1) (Leroith *et al*., 2004)

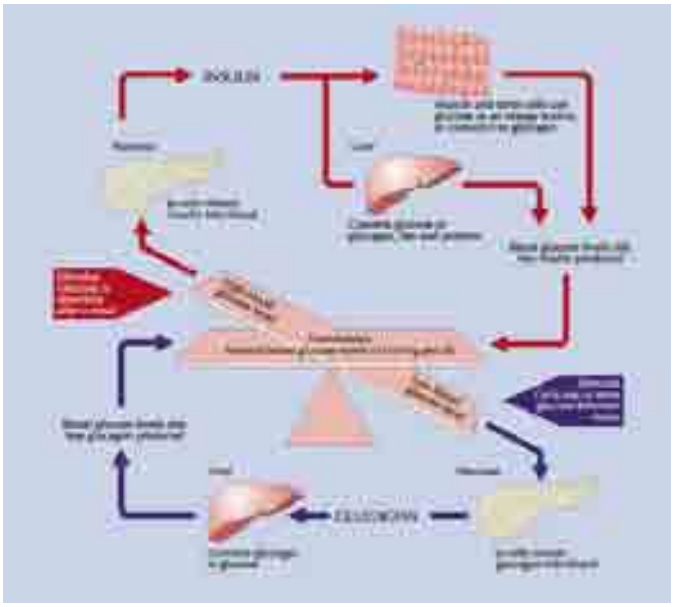


Figure 1.1 Mechanism of diabetes mellitus.

1.1 Diabetes mellitus: Classifications, causes and its complication

DM is classified into two types : type 1 or insulin-dependent diabetes mellitus (IDDM) and type 2 or non-insulin-dependent diabetes mellitus (NIDDM) (Figure 1.2).

Figure 1.2 Defective insulin secretion for type I and II diabetes (www.uscf.mightyminnow.com/images/charts).

Type 1 DM is characterized by loss of insulin producing of pancreatic β-cell islets, leading to a deficiency of insulin. The metabolism of glucose regulation enters bloodstream in this type is show in Figure 1.2. Type 1 diabetes usually appears before the age of 40. This type of diabetes is the least common of the two main types and accounts for between 5 – 15% of all people with diabetes. Type 1 diabetes has been postulated that environmental factors such as certain viral infections and possibly chemical or nutritional agents may worsen these genetic factors (Leroith *et al*., 2004).

Type 2 DM is characterized by the body can still secret some insulin, but not enough, or when the insulin that is produced does not work properly (known as insulin resistance) as show in Figure 1.2. Insulin secretion from the pancreas normally reduces glucose output by the liver, enhances glucose uptake by skeletal muscle, and suppresses fatty acid release from fat tissue. The various factors shown that contribute to the pathogenesis of type 2 diabetes affect both insulin secretion and insulin action. Decreased insulin secretion will reduce insulin signaling in its target tissues. Insulin

resistance pathways affect the action of insulin in each of the major target tissues, leading to increased circulating fatty acids and the hyperglycemia. In turn, the raised concentrations of glucose and fatty acids in the bloodstream will feed back to worsen both insulin secretion and insulin resistance (Leroith *et al*., 2004).

Type 2 DM is the most common type of diabetes, accounting for 90 to 95 percent of all diabetes. It usually develops after the age of 40. However, in the late 1990's, its incidence increased among young people. Experts are trying to determine why that is happening. It may be related to the increased incidence of obesity and sedentary lifestyles among young people. There are currently over 3 million people with diabetes in Thailand and there are more than half a million people with diabetes who have the condition and do not know it. About 80 percent of those with type 2 diabetes are overweight. It is more common among people who are older, sedentary or obese, or have a family history of the disease. It may reappear in women who had gestational diabetes. It is more common among people of Asian, Hispanic, African or Native American ancestry.

Type 2 DM is a progressive disease that can cause significant, severe complications such as heart disease, kidney disease, blindness and loss of limbs through amputation. Treatment differs at various stages of the condition. In its early stages, many people with type 2 diabetes can control their blood glucose levels by losing weight, eating properly and exercising. Many may subsequently need oral medication, and some people with type 2 diabetes may eventually need insulin shots to control their diabetes and avoid the disease's serious complications. Even though there is no cure for diabetes, proper treatment and glucose control enable people with type 2 diabetes to have normal and productive lives (Loutfi *et al*., 2003).

1.2 α-Glucosidase inhibitors

Treatment of type 2 diabetes has several approaches that diverge at many stages of condition. In the therapeutic stage, many patients can control blood glucose levels by dietary, exercise and weight loss. In addition to therapeutic approach, the

effective approach is to delay the postprandial hyperglycemia. This can be achieved by retarding the absorption of glucose through the inhibition of the carbohydrate-hydrolyzing enzymes α-glucosidase (sucrase, maltase and isomaltase) in the digestive process of the small intestine (Figure 1.3). Inhibtitors of these enzyme delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose level (Rhabasa-Lhoret *et al*., 2004). Furthermore, the α-glucosidase inhibitors decrease the posprandial increment in plasma insulin levels, reducing triglyceride levels and anti-HIV activity (Bridges *et al*., 1994; Fischer *et al*., 1996). Currently, the α-glucosidase inhibitors are used orally as antidiabetes including acarbose (Precose® or Glucobay®), miglitol (Glyset®) and voglibose (Basen®) (Melo *et al.*, 2006).

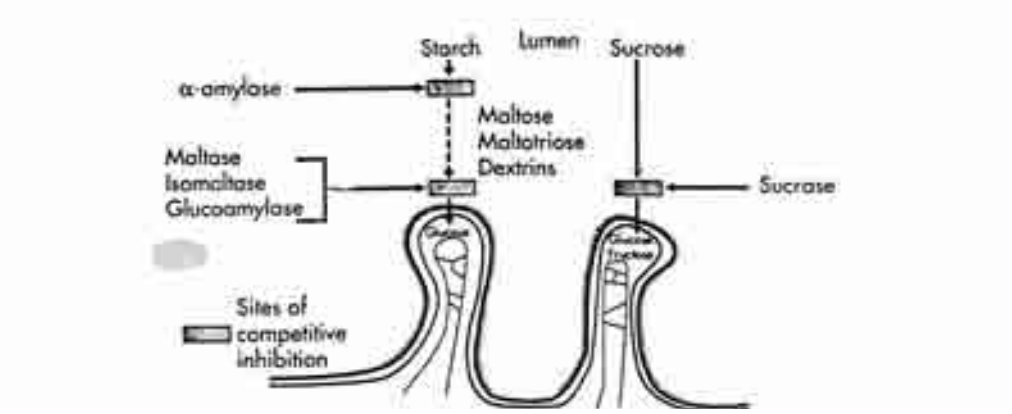
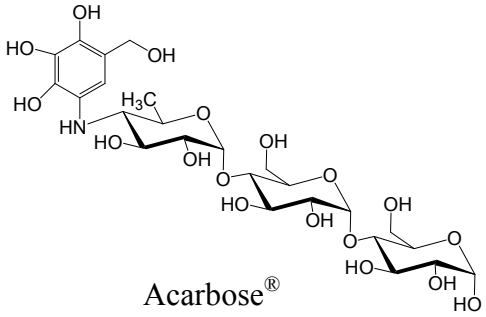


Figure 1.3 In normal digestion, pancreatic α-amylase hydrolyzes complex starches into oligosaccharides, which are further hydrolyzed by α-glucosidase located in the intestinal brush border to glucose and other monosaccharides, which are then absorbed.

Acarbose (Precose® or Glucobay®) has been produced as a secondary metabolite on a large scale from fermentation cultures of *Actinoplanes sp*. Catalytic hydrogenation of acarbose afforded fragments consisting of trisaccharide derivative, which have inhibitory activity on α-glucosidase and significantly decreases the postprandial increase in plasma glucose after the ingestion of mixed carbohydrate

meal without changing the total amount of carbohydrate absorbed (Figure 1.4) (Bischoff *et al*., 1994). Importantly, therapeutic doses of acarbose® do not cause malabsorption, but long-term acarbose® administration was side effect such as flatulence, bloating, diarrhea and soft stools.



Acarbose®

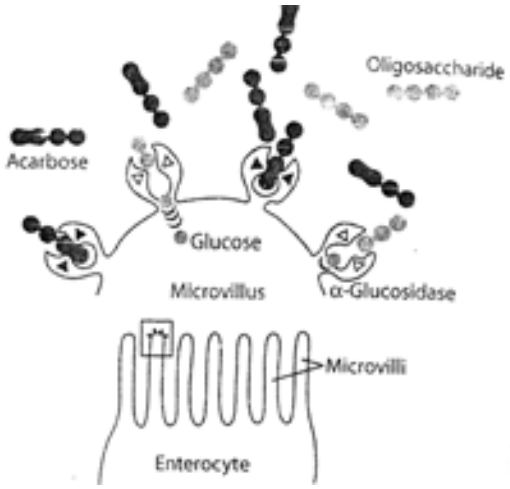


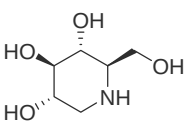
Figure 1.4 Acarbose competitively inhibits the enzymatic hydrolysis of oligosaccharide by α-glucosidase in the small intestine.

1.3 Antidiabetes drugs from medicinal plants

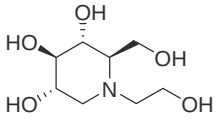
In many developing countries , the use of herbal medicine by the suffers of chronic disease is encouraged because there is concern about the adverse effects of chemical drugs and treatment using medicines of natural origin appears to offer more gentle means of managing such disease (Bhattarai, 1993; Manandham, 1995; Shrestha and Joshi, 1993). Herbal drugs are prescribed widely because of their effectiveness,

fewer side effect and relatively low cost. To this end, research has begun to embrace traditional medicines from various cultures, as scientists search for clues to discover new therapeutic drugs for diabetes (Li *et al*., 2004). Traditional Indian and Chinese medicine have long used plant and herbal extracts as anti-diabetic agents (Chen *et a*l., 2001; Grover *et al*., 2002). Therefore, investigation on such agents from traditional medicinal plants has become more important and researches are competing to find the new effective and safe therapeutic agent for the treatment of diabetes.

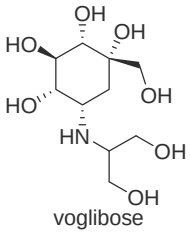
A recent review of antihyperglycemia compounds mentions the following plants with α-glucosidase activity such as 1-deoxynojirimycin (DNJ), which was isolated from *Morus alba* (Singab *et al*., 2005). It had inhibitory effect against α-glucosidase. However, the activity of DNJ in vivo against intestinal sucrase was lower than that observed in vitro, and this initiated a synthesis to produce derivatives with enhanced activity. The *N*-alkyl derivatives were most effective and this led to the development of *N*-hydroxyethyl deoxynojirimycin (known as Miglitol or Glyset®) as an oral treatment of the type 2 diabetes (Melo *et al*., 2006).



DNJ



miglitol

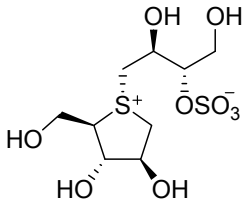


voglibose

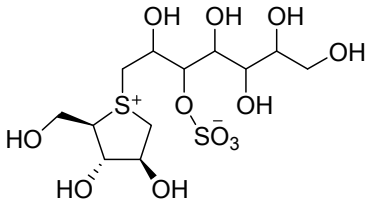
Volglibose (Basen®) can be regarded as derivative of 1-deoxynojirimycin (DNJ), which also has a high inhibitory activity against sucrase and maltase. It has been employed in Japan for the treatment of diabetes since 1994. In recent studies based on α-glucosidase inhibitory activity, it was shown to be 20 to 30 times more potent than acarbose, thus increasing glucose tolerance by inhibiting its digestion and absorption in the intestine, especially after meals (Yasuda *et al*., 2003). Additionally, the use of voglibose led to less adverse effects including flatulency and abdominal distention, as shown in a random comparative study (Melo *et al*., 2006).

Salacinol was islolated from an aqueous extract of the roots and stems of *Salacia reticulata* Wight (Yoshikawa *et al*., 1997), which has been traditionally used

in India and Sri Lanka for the treatment of diabetes. Salacinol displayed a strong inhibition for the increase of serum glucose levels in vivo screening along with competitive inhibition against intestinal α-glucosidase such as maltase, sucrase and isomaltase; in which the activity against isomaltase was higher than that of acarbose. Kotalanol, a derivative of 1,2,3-trihydroxypropylsalacinol, showed more potent inhibitory activity against sucrase than salacinol and acarbose (Yoshikawa *et al*., 1998). Kotalanol was developed to a diabetic drug that used generally in name Diabosol®. A recent study in healthy adults (Heacock, 2005) showed significant reduction of postprandial plasma glucose, serum insulin and increased breath hydrogen after ingestion of 1000 mg of *Salacia oblonga* extract. The increase in breath hydrogen is attributable to a mechanism involving inhibiton of α-glucosidase.



salacinol

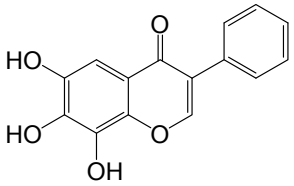


kotalanol

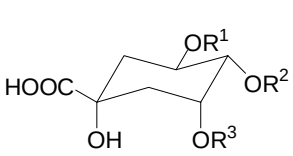
Pycnogenol®, the standardized maritime pine bark extract derived from *Pinus pinaster*, has been reported to display antidiabetes effect in patient (Liu *et al*., 2004a and 2004b). Supplementation with 100 mg for 3 months significantly lowered blood glucose levels compared to Placebo, and improved endothelial function was observed in type 2 diabetic patients. A recent study revealed that Pycnogenol®, which mainly contained proxyanidin oligomers, potentially inhibited α-glucosidase with IC_{50} value of 5 μg/mL (Schäfer and Högger, 2007).

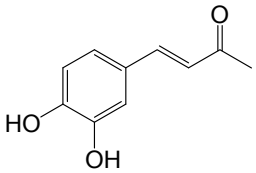
In addition, compounds possessing antidiabetic activity include flavonoids (flavonones, flavones, chalcoes and their glycosides), xanthones and polyphenols. They were exemplified by the reports of baicalein (5,6,7-trihydroxy flavone) from the root of *Scutellaria baicalensis* and its 6-hydroxy analogues from *Origanum majorana.* Baicalein strongly inhibited sucrase (IC_{50} = 52 μM) while its inhibitory effect against maltase was moderate (IC_{50} = 500 μM) (Nishioka *et al*., 1998; Kawabata *et al*., 2003).

Investigation on structure-activity relationship among different flavones derivatives indicated that the loss of hydroxyls from positions 5, 6, and 7 significantly reduced the activity. Some polyphenols, 3, 5-dicaffeoyl-quinic acid, 4,5-dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid, were found in the flower buds of *Tussilago farfara* L. These three compounds showed comparative inhibitory activities against maltase (Gao *et al*., 2007). A recent study reported that xanthones were capable of inhibiting α-glucosidase with moderate to high activities. Promonent instances included isoprenyl tetrahydroxy xanthones isolated from the roots of *Cudrania tricuspidata,* which possessed highly potent α-glucosidase inhibition with IC_{50} value in range of 16.2-52.9 μM (Seo *et al*., 2007). In addition, mangiferin, a xantone from *Swertia chirayita*, redued blood glucose levels in STZ-induced diabetic rats (Muruganandan, 2002).



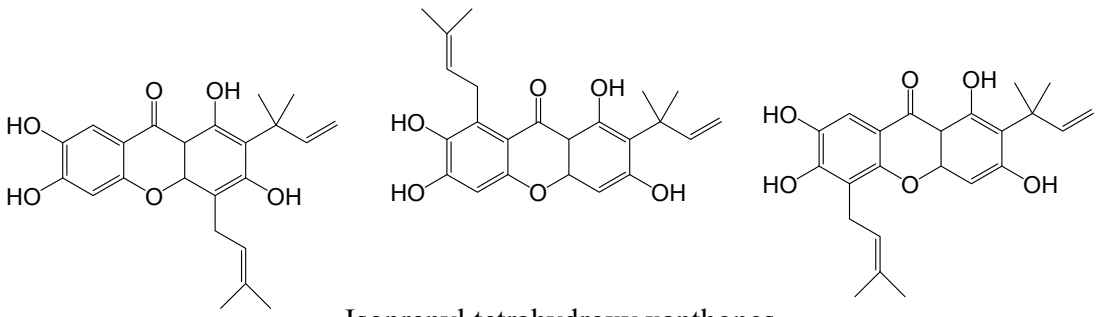
Baicalein



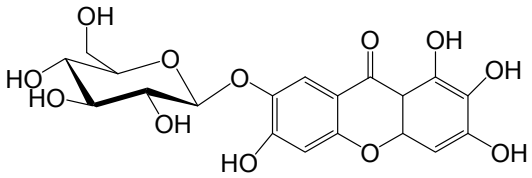


Caffeoyl

3,4-Dicaffeoylquinic acid : R^1= H, R^2 = R^3 = caffeoyl
3,5-Dicaffeoylquinic acid : R^1= R^3 = caffeoyl, R^2 = H
4,5-Dicaffeoylquinic acid : R^1= R^2 = caffeoyl, R^3 = H



Isoprenyl tetrahydroxy xanthones



Mangiferin

Figure 1.5 Structures of selected natural products having antidiabetic properties.

Thai etnopharmacology, the knowledge of medicinal plants is abundant within the Thai Traditional Medicine. Thai Traditional Medicine was long-term mainstream medical system, which has benefit of the healthcare system. In addition, there are many reports showed increasing use of medicinal plants as antidiabetic drugs, which studied both in subject and animal models. Therefore, Thai medicinal plants have potentially antidiabetic.

Criterion of selectively Thai medicinal plants

1.Reducing blood sugar and no toxic in long-term administration

2.Commercially or readily available and use less time in production

3.No report on the use as α-glucosidase inhibitors

In this research, we interested that hypoglycemic in *M.oleifera* and *S.indicum*.

CHAPTER II

α-GLUCOSIDASE INHIBITOR FROM *Moringa oleifera* Lam. Leaves

2.1. Introduction

2.1.1 Botanical aspect and distribution of *Moringa oleifera*

Moringa oleifera belongs to family Moringaceae, which is commonly known as horseradish tree, drumstick tree, benzolive monga and in Thai as Ma Rum. The leaves, fruit, flowers and immature pods are used as a highly nutritive vegetable in many countries, particularly in Asia, Africa, Thailand and other tropical parts of the world as a vegetable for cooking purposes (Shaheen *et al*., 1995). It is a multipurpose tree widespread throughout most of the tropics (Oliveira *et al*., 1999). It is a rapidly-growing ornamental tree and reached up to 12 m. The bark is grey and thick and looks like cork, peeling in patches. It loses its leaves from December to January and new growth starts in February to March. *Moringa* produces cream colored flowers when it is 8 months old and the flowering season begins in January and continues through to March. The fruit ripens from April to June and the pods are triangular in cross section, 30 to 50 cm long and contain oily, black, winged seeds. It is a perennial softwood tree with timber of low quality, but which for centuries has been advocated for traditional medicine and industrial uses (Fahey *et al*., 2005).

Figure 2.1 *Moringa oleifera*.

2.1.2 Pharmacological and phytochemical investigation of *Moringa oleifera*

M.oleifera is especially promising as a food source in the tropic. This plant has an impressive range of medicinal uses with high nutrition value that different parts of its contain a profile of important minerals, and a good source of protein, vitamins, *β*-carotene, amino acids and various phenolics (Anwar *et al*., 2007 and Fahey *et al*., 2005). The leaves have been reported to be a rish source of *β*-carotene, protein, vitamin C, moisture, carbohydrate, magnesium, iron, calcium, potassium and act as a good source of natural antioxidants (Anwar *et al*., 2007 and Yameogo *et al*., 2011). In addition, the leaves can be a food available all year round and to have high quality for the men (Yameogo *et al*., 2011)

Pharmacological properties and medicinal uses, *M.oleifera* is very important for its medicinal value. Various part of this plant act as cardiac and circulatory stimulants, possess antitumor, antipyretic, anti-inflammatory, antiepileptic, hepatoprotective, antioxidant, antihypertensive, cholesterol lowering and antidiabetic. In addition, medicinal properties have been arbitrated to diverse parts of this highly regarded tree (Table 2.1) and reviewed by Anwar *et al*., 2007.

Antidiabetic property of *M.oleifera* in animal model was reported. Francis and coworker investigated insulin secretagogues from *M.oleifera*, in addition to cyclooxygenase enzyme and lipid peroxidation inhibitory activities. They found that phenolic rhamnosides named 1-*O*-phenyl-α-L-rhamnopyranoside and methyl *N*-{4-[(α-L-rhamnopyranosyl)benzyl]}carbamate significantly stimulated insulin release (ca. 30 mg/mg protein) in rat pranceatic β-cells. This study indicated antidiabetic potential of *M.oleifera* as sulfonylurea, an insulin-releasing stimulator (Francis *et al*., 2004)

Jaiswal and coworker studied the effect of leave aqeous extract on blood glucose levels of normal and hyperglycemic rats. The extract reduced the blood glucose level in normal rat and normalized high blood glucose levels in sub, mild and severely diabetic rats within 3 h. In addition, It improved glucose tolerance both in normal and hyperglycemic rat more effectively than reference drug Glipizide (Jaiswal *et al*., 2009).

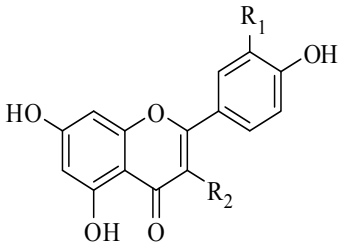
In our project, we hypothesize that hypoglycemic effect of *M.oleifera* is possibly linked to α-glucosidase inhibitory activity, in addition to insulin-releasing

stimulation. Therefore, this research is aimed at identifying active compound in this plant.

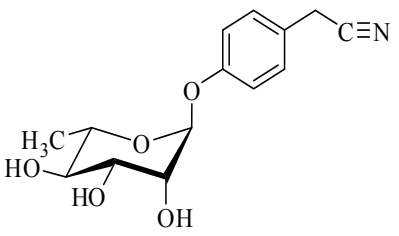
Phytochemistry of leaves, containing two major compounds, which is phenolic glycoside (almost sugar moiety as rhamnose) and flavonoid. The major compound were found to be flavonoid group such as kaempferol and quercetin (Siddhuraju and Becker, 2003). However, the structure of selected phytochemicals from *Moringa oleifera* leaves was shown in Figure 2.2 and reviewed by Anwar *et al*., 2007; Bennett *et al*., 2003 and Sahakitpichan *et al*., 2011

Table 2.1 Some common medicinal uses of different parts of *Moringa oleifera*

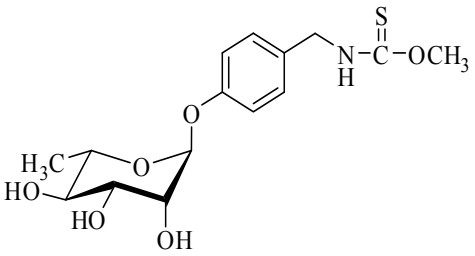
Plant part	**Medicinal Uses**	**Reference**
Root	Anti-inflammatory, antilithic, rubefacient, vesicant, antifertility, carminative, treating rheumatism, articular pains, constipation and car minative.	Padmarao, P. *et al*., 1996; Dahot, M. U. *et al*., 1988; Ruckmani, K. *et al*., 1998.
Leave	Purgative, used for fever, piles, sore throat, eye and ear infection, applied to reduce glandular and treatment of diabetes mellitus.	Dahot, M. U. *et al*., 1988; Makonnen, E. *et al*., 1997.
Stem bark	Rubefacient, prevent enlargement of the spleen and formation of tuberculous glands of the neck,to destroy tumors and anti-tubercular activity.	Bhatnagar, S. S. *et al*., 1961; Siddhuraju, P. *et al*., 2003.
Gum	Relieved headaches, fevers, intestinal complaints, dysentery, asthma and treated syphilis and rheumatism and used for dental caries.	Fuglie, L. J. *et al*., 2001.
Flower	Treated inflammation, muscle disease, tumors, lower serum cholesterol, phospholipid, triglyceride, decrease lipid profile of liver, heart and aorta in hypercholesterolaemic rabbits.	Bhattacharya, S. B. *et al*., 1982; Dahot, M. U. *et al*., 1998; Siaddhuraju, P. *et al*., 2003; Mehta, L. K. *et al*., 2003.
Seed	Decreasing liver lipid peroxides, antihypertensive compounds thiocarbamate and isothiocyanate glycosides have been isolated from the acetate phase of the ethanolic extract of Moringa pods	Faizi, S. *et al*., 1998; Lalas, S. *et al*., 2002.



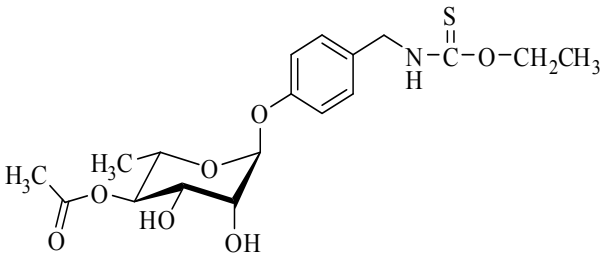
	R1	R2
kaempferol 3-O-glucoside	H	Glc
kaempferol 3-O-(6''-malonylglucoside)	H	Glc-6"-O-CO-CH_2-COOH
quercetin 3-O-glucoside	OH	Glc
quercetin 3-O-(6"-malonylglucoside)	OH	Glc-6"-O-CO-CH_2-COOH
quercetin 3-O-rhamnosylglucoside (Rutin)	OH	Glc-Rham



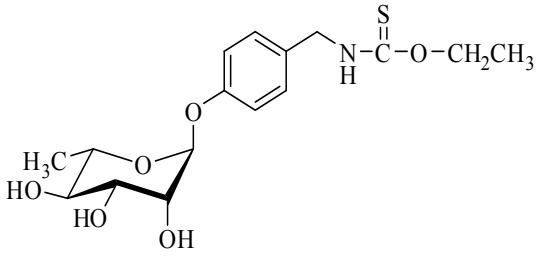
niazirin



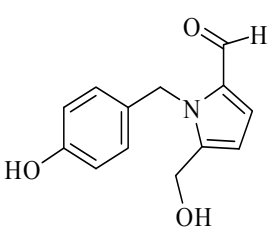
niazinin



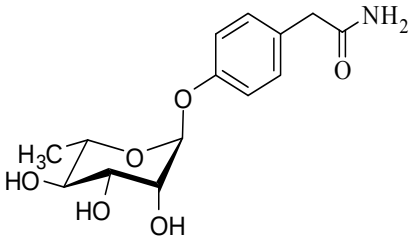
niaziminin



niazimicin



pyrrolemarumine

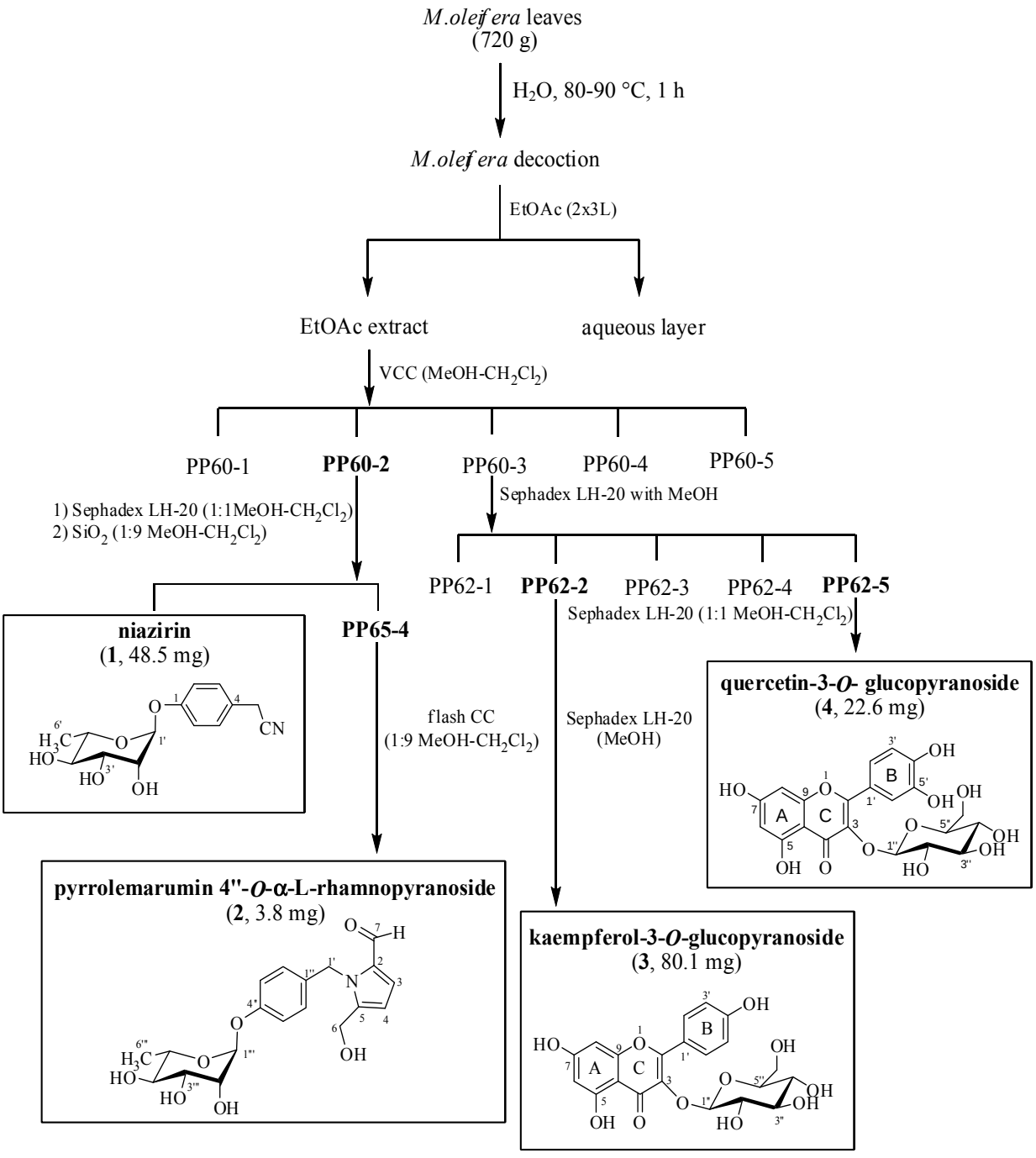


4'-hydroxyphenylethanamide-α-L-rhamnopyranoside

Figure 2.2 Structures of selected phytochemicals from *Moringa oleifera*

2.2 Isolation

The air-dried leaves of *Moringa oleifera* (720 g) were boiled with H_2O at 80-90 °C for 1 h and extracted with EtOAc for 2 times to obtain EtOAc extract and aqueous layer. The EtOAc extract was evaporated to dryness and separated through vacuum column chromatography using stepwise EtOAc-hexane (1:1, 4:1), EtOAc, 1:9 MeOH-EtOAc and MeOH, respectively, yielding five major fractions (PP60-1 to PP60-5). Fraction PP60-2 was separated through sephadex LH-20 column using 1:1 MeOH-CH_2Cl_2 followed by silica gel CC using 1:9 MeOH-CH_2Cl_2 to yield four subfractions. Subfraction PP65-3 afforded nitrile glycoside named niazirin (**1**). Subfraction PP65-4 was purified by flash column chromatography using 1:9 MeOH-CH_2Cl_2 to afford alkaloid glycoside named pyrrolemarumine 4"-*O*-α-L-rhamnopyranoside (**2**). Fraction PP60-3 was purified by sephadex LH-20 column using MeOH to obtain five subfractions. Subfraction PP62-2 was purified by sephadex LH-20 column with MeOH to afford flavoniod glycosides named kaempferol-3-*O*-*β*-glucopyranoside (**3**). Subfraction PP62-5 was purified by sephadex LH-20 column using 1:1 MeOH-CH_2Cl_2 to afford quercetin-3-*O*-*β*-glucopyranoside (**4**) (Scheme 2.1 and Figure 2.3).



Scheme 2.1 Isolation procedure of *Moringa oleifera* leaves.

2.3 Structure elucidation of isolated compounds 1-4

Compound **1** was obtained as a yellow solid. The structure was deduced by the results from ^{1}H, ^{13}C and confirmed with 2D NMR spectroscopic methods. The ^{1}H NMR showed characteristic signal of para-disubstituted aromatic ring [δ_H 6.98 (2H, d, *J* = 8.8 Hz, H-2 and H-6) and 7.19 (2H, d, J = 8.4 Hz, H-3 and H-5)]. The singlet proton at δ_H 3.73 as the methylene group (H-7). The sugar unit, suggest to be an α-L-rhamnopyranosyl moiety were revealed anomaric and secondary methyl protons at δ_H 5.33 (1H, d, *J* = 1.2 Hz) and 1.12 (3H, d, *J* = 6.4 Hz), respectively. The ^{13}C NMR dedicated 1, 4-disubstituted benzene at δ_C 156.0 and 124.4. In addition, cyanide was showed signal at δ_C 118.4. δ_C 98.3 and 16.6 were assign as anomaric and secondary methyl carbons, respectively. Therefore, the structure of compound **1** as the same structure of Niazirin (Faizi *et al.,* 1994).

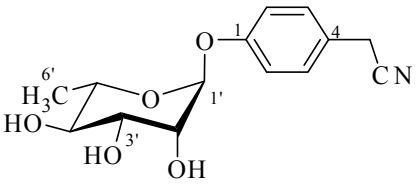


Figure 2.3 niazirin (**1**)

Compound **2** was obtained as a pale yellow amorphous powder. The structure was deduced by the results from ^{1}H, ^{13}C and confirmed with 2D NMR spectroscopic methods. The ^{1}H NMR dedicated characteristic signal of nitrogen-containing heterocyclic ring [δ_H 6.96 and 6.24 (each 1H, d, *J* = 4 Hz, H-3 and H-4)]. The singlet proton signal at δ_H 9.35 were showed aldehyde group. Two singlet methylene protons at δ_H 4.43 and 5.57 were assign as H-6 and H-1', respectively. In addition, the sugar moiety, suggest to be an α-L-rhamnopyranosyl moiety were revealed animaric and secondary methyl protons at δ_H 5.27 (1H, d, *J* = 1.2 Hz) and 1.10 (2H, d, *J* = 6 Hz). The ^{13}C NMR showed characteristic two para-disubstituted, which is 2, 5-disubstituted pyrrloe ring (δ_C 132.2 and 143.6, H-2 and H-5) and 1, 4-disubstituted aromatic ring (δ_C 131.8 and 155.5, H-1" and H-4"). Aldehyde carbon at δ_C 179.9 and two methylene carbons at 55.5 and 49.0 were assign as H-7, H-6 and H-1", respectively. δ_C 98.2 and 16.6 (H-1'" and H-6'") were assign as animaricand secondary methyl carbons. Thus, the structure of compound **2** as the same structure of pyrrolemarumine 4"-*O*-α-L-rhamnopyranoside (Sahakitpichan *et al.,* 2011).

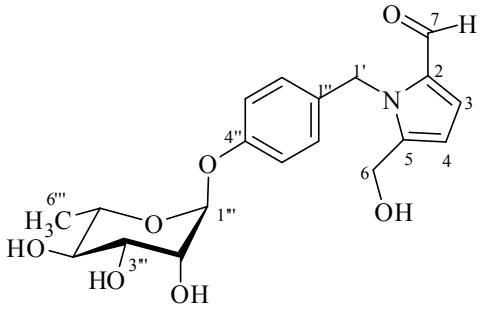


Figure 2.4 pyrrolemarumine 4"-*O*-α-L-rhamnopyranoside (**2**)

Compound **3** was obtained as a yellow solid. The structure was deduced by the results from ^{1}H, ^{13}C NMR spectroscopic methods. The ^{1}H NMR spectral showed, For aromatic ring, Two doublet signals at δ_H 7.96 (H-2' and H-6') and 6.79 (H-3' and H-5'), Both of which integrated for two protons, were dueto protons of the B ring. These proton signals displayed as two pair of the ortho couple doublet with J = 8.8 Hz and H-6 and H-8 gave two meta-coupled doublets at δ_H 6.11 and 6.31 (J = 2 Hz) were due to protons of the A ring. The sugar unit, suggested to be an 3-*O*-glucopyranosyl moiety were revealed by the corresponding anomaric proton at δ_H 5.17 (d, J = 7.6 Hz). Therefore, the structure of compound **3** as the same structure of kaempferol-3-*O*-*β*-glucopyranoside (Lin *et al.,* 2009).

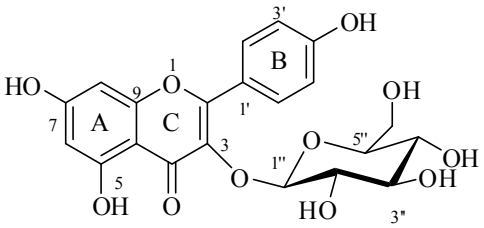


Figure 2.5 kaempferol-3-*O*-*β*-glucopyranoside (**3**)

Compound **4** was obtained as a yellow solid. The structure was deduced by the results from ^{1}H, ^{13}C NMR spectroscopic methods. The ^{1}H NMR spectral showed, For aromatic side, The signals at δ_H 7.61 (d, J = 2 Hz), 6.78 (d, J = 8.4 Hz) and 7.49 (dd, J = 8.4 Hz) could be ascribed to H-2'. H-5' and H-6', respectively, were characterized to B ring. Two doublet proton signals at δ_H 6.12 and 6.31 (J = 2 Hz) could be ascribe to H-6 and H-8, respectively, gave two meta-coupled. The sugar unit, suggest to be an 3-*O*-glucopyranosyl moiety were revealed by the corresponding anomaric proton at δ_H 5.16 (d, J = 7.6 Hz). Therefore, the structure of compound **4** as the same structure of quercetin-3-*O*-*β*-glucopyranoside (Lin *et al.,* 2009).

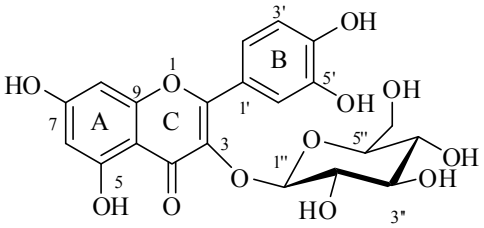


Figure 2.6 quercetin-3-*O*-*β*-glucopyranoside (**4**)

2.4 α-Glucosidase inhibitory activity of the isolated compounds

The α-glucosidase inhibitory activity of compound **1-4** isolated from *Moringa oleifera* leaves was evaluated by colorimetric method and the results are shown in Table 2.2

Table 2.2 α-glucosidase inhibitory activity of isolated compounds from *Moringa oleifera* leaves

compounds	IC_{50} (μM) [a]		
	α-glucosidase		
	Baker's yeast	Rat small intestine	
		maltase	sucrase
1	27.23	2783.13	4478.52
2	81.67	779.27	1865.84
3	NI[b]	74.12	346.48
4	NI	95.73	54.80
acarbose®	220.55	1.50	2.38

[a] The IC_{50}value is defined as the inhibitor concentration to inhibit 50% of enzyme activity.
[b] No inhibition, less than 50% inhibition at 10 mg/ml

From Table 2.2 showed that the phenolic rhamnosides **1** and **2** selectively inhibited α-glucosidase from baker's yeast, whereas flavonoid glycosides **3** and **4** selectively inhibited that from rat intestine. For phenolic rhamnosides, niazirin (**1**) is the most potent α-glucosidase inhibitor against baker's yeast, which is approximately 8 times higher than that of antidiabetic drug acarbose. On the other hand, the series of flavonoid glycosides exhibited strong inhibitory activity toward rat intestinal α-glucosidase. Quercetin (**4**) inhibited maltase (IC_{50} 95.73 μM) and sucrase (IC_{50} 54.80 μM) more potent than kaempferol (**3**); while both of them were inactive against α-glucosidase from baker's yeast.

These remarkable results revealed that the series of phenolic rhamnosides **1** and **2** displayed the strong interaction with the amino acid residues of the active site of baker's yeast α-glucosidase. For this data, it is proposed that nitrogen atom in their molecular structures played important role for enzyme inhibition. Moreover, there have not been reported on the α-glucosidase inhibitory activity of them, therefore, in this research reported for the first time. As for flavonoid glycosides **3** and **4**, their interesting data suggested that the presences of hydroxyl groups on aromatic ring can form hydrogen bonds with the polar groups of rat intestinal α-glucosidase. Thus, α-glucosidase from human and rat intestines are nearly similar in active site, we therefore conclude that quercetin (**4**) is a putative α-glucosidase inhibitor responsible for hypoglycemic effect of *M. oleifera*.

2.5 Experiment section

2.5.1 General experiment procedures

The ^{1}H and ^{13}C NMR spectra (in $CDCl_3$, CD_3OD and acetone-d_6) were determined with a nuclear magnetic resonance spectrometer of Varian model Mercury+ 400. The chemical shift in δ (ppm) was assigned with reference to the signal from the residual protons in deuterated solvents and using TMS as an internal standard in some cases. ESIMS was obtained from Mass Spectrometer Model VG TRIO 2000. Sephadex LH-20 and silica gel 60 Merck cat. No. 7734 and 7729 were used for open column chromatography. Thin layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm thick layer).

2.5.2 Plant material

The leaves of *M.oleifera* (voucher's specimen number: BCU 013507) were collected in Lampang, Thailand in January 2010

2.5.3 Extraction and isolation

The air-dried leaves of *M.oleifera* (720 g) were boiled with H_2O at 80-90 °C for 1 h and extracted with EtOAc for 2 times to obtain EtOAc extract and aqueous

layer. The EtOAc extract was evaporated to dryness and separated through vacuum column chromatography using stepwise EtOAc-Hexane (1:1, 4:1), EtOAc, 1:9 MeOH-EtOAc and MeOH, respectively, yielding five major fractions (PP60-1 to PP60-5). Fraction PP60-2 was separated through sephadex LH-20 column with 1:1 MeOH-CH_2Cl_2 followed by silica gel CC with 1:9 MeOH-CH_2Cl_2 to yield four subfractions. Subfraction PP65-3 afforded nitrile glycoside named Niazirin (**1**, 48.5 mg). Subfraction PP65-4 was purified by flash column chromatography with 1:9 MeOH-CH_2Cl_2 to afford glycoside of pyrrolealkaloid named pyrrolemarumine 4"-*O*-α-L-rhamnopyranoside (**2**, 3.8 mg). Fraction PP60-3 was purified by sephadex LH-20 column with MeOH to obtain five subfractions. Subfraction PP62-2 was purified by sephadex LH-20 column with MeOH to afford flavoniods glycoside named kaempferol-3-*O*-*β*-glucopyranoside (**3**, 80.1 mg). Subfraction PP62-5 was purified by sephadex LH-20 column with 1:1 MeOH-CH_2Cl_2 to afford quercetin-3-*O*-*β*-glucopyranoside (**4**, 22.6 mg).

Niazirin (**1**) as Lemon Yellow solid; ^{1}H NMR (400 MHz., methanol-d_4) δ 7.19 (2H, d, *J* = 8.4 Hz, H-3 and H-5), 6.98 (2H, d, *J* = 8.8 Hz, H-2 and H-6), 5.33 (1H, d, *J* = 1.2 Hz, H-1'), 3.90 (1H, dd, *J* = 1.2 Hz, H-2'), 3.73 (2H, s, H-3'), 3.52 (1H, m, H-5'), 3.38 (1H, m, H-4'), 1.12 (3H, d, *J* = 6.4 Hz, H-6').^{13}C NMR (400 MHz, methanol-d_4) δ 156.0 (C-1), 128.9 (C-3 and C-5), 124.4 (C-4), 118.4 (C-8), 116.6 (C-2 and C-6), 98.3 (C-1'), 72.5 (C-4'), 70.7 (C-3'), 70.6 (C-2'), 21.3 (C-7), 16.6 (C-6').

Pyrrolemarumine 4"-*O*-α-L-rhamnopyranoside (**2**) as Pale Yellow solid; ^{1}H NMR (400 MHz., MeOH-d4) δ 9.35 (1H, s, H-7), 6.96 (1H, d, *J* = 4 Hz, H-3), 6.87 (4H, m, H-2", H-3", H-5" and H-6"), 6.24 (1H, d, *J* = 4 Hz, H-4), 5.57 (2H, s, H-1'), 5.27 (1H, d, *J* = 1.2Hz, H-1'"), 4.43 (1H, s, H-6), 3.72 (1H, d, *J* = 3.6 Hz, H-2'"), 3.70 (1H, d, *J* = 3.6 Hz, H-3'"), 3.51 (1H, q, *J* = 3.6 Hz, H-5'"), 3.35 (1H, t, *J* = 9.6 Hz, H-4'"), 1.10 (2H, d, *J* = 6 Hz, H-6'"),.^{13}C NMR (400 MHz, methanol-d_4) δ 179.9 (C-7), 155.5 (C-4"), 124.4 (C-4), 143.6 (C-5), 132.2 (C-2), 131.8 (C-1"), 127.1 (C-2",6'), 125.0 (C-3), 116.2 (C-3" and C-5"), 110.0 (C-4), 98.2 (C-1'"), 72.4 (C-4'"), 70.7 (C-2'" and C-3'").

Kaempferol-3-*O*-*β*-glucopyranoside (**3**) as a yellow solid ; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.96 (2H, d, *J* = 8.8 Hz, H-2' and H-6'), 6.79 (2H, d, *J* = 8.8 Hz,

H-3' and H-5'), 6.31 (1H, d, *J* = 2 Hz, H-8), 6.11 (1H, d, *J* = 2 Hz, H-6), 5.17 (1H, d, *J* = 2 Hz, H-1''), 3.61-3.10 (sugar moiety). ^{13}C NMR (400 MHz, methanol-d_4) δ 177.4, 164.1, 161.1, 159.9, 156.3, 156.2, 133.1, 130.8, 130.8, 120.8, 115.1, 115.1, 103.9, 100.8, 98.6, 93.6, 77.4, 76.4, 74.1, 69.8, 60.8. ^{13}C NMR (400 MHz, methanol-d_4) δ 177.4, 164.1, 161.1, 159.9, 156.3, 156.2, 133.1, 130.8, 130.8, 120.8, 115.1, 115.1, 103.9, 100.8, 98.6, 93.6, 77.4, 76.4, 74.1, 69.8, 60.8.

Quercetin-3-*O*-*β*-glucopyranoside (**4**) as yellow solid; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.61 (1H, d, *J* = 2 Hz, H-2'), 7.49 (1H, dd, *J* = 8.4 Hz, H-6'), 6.78 (1H, d, *J* = 8.4 Hz, H-5'), 6.31 (1H, d, *J* = 2 Hz, H-8), 6.12 (1H, d, *J* = 2 Hz, H-6), 5.17 (1H, d, *J* = 7.6 Hz, H-1''), 3.64-3.12 (sugar moiety). ^{13}C NMR (400 MHz, methanol-d_4) δ 177.7, 165.0, 162.0, 158.0, 158.0, 148.0, 144.0, 134.0, 121.8, 121.4, 116.1, 114.6, 104.0, 102.8, 98.5, 93.3, 77.0, 76.6, 74.3, 69.7, 61,1.

2.5.4 α-Glucosidase inhibitory assay

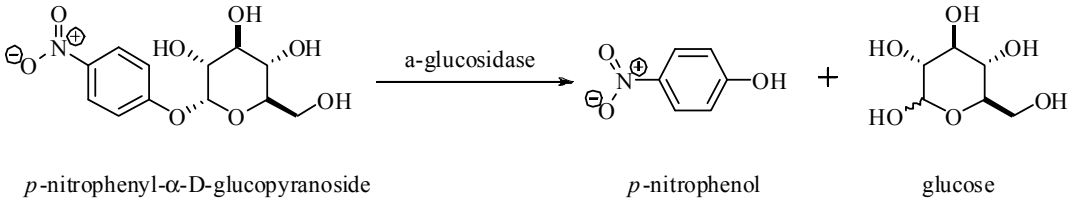
2.5.4.1 Chemical and equipment

Sucrose, maltose, baker's yeast α-glucosidase, rat intestinal acetone powder, and *p*-nitrophenyl-α-D-glucopyranoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glucose assay kit was purchased from Human Gesellschaft für Biochemica und Diagnostica mbH (Germany). Acarbose was obtained from Bayer (Germany).

2.5.4.2 Baker's yeast α-glucosidase inhibitory activity

The α-glucosidase inhibition assay was performed according to the slightly modified method of Wacharasindhu *et al*. The α-glucosidase (0.1 U/mL) and substrate (1 mM *p*-nitrophenyl-α-D-glucopyranoside) were dissolved in 0.1 M phosphate buffer, pH 6.9. 10 μL of synthesized compounds (1 mg/mL in DMSO) was pre-incubated with 40 μL of α-glucosidase at 37 ·C for 10 min. A 50 μL substrate solution was then added to the reaction mixture and incubated at 37 ·C for 20 min, and terminated by adding 100 μL of 1 M Na_2CO_3. Enzymatic activity was quantified by measuring the absorbance at 405 nm (Bio-Red microplate reader model 3550 UV). The percentage inhibition was calculated by $[(A_0-A_1)/A_0] \times 100$, where A_0 is the absorbance without the sample, and A_1 is the absorbance with the sample. The IC_{50}

value was determined from a plot of percentage inhibition versus sample concentration. Acarbose® was used as standard control and the experiment was performed in duplicate.

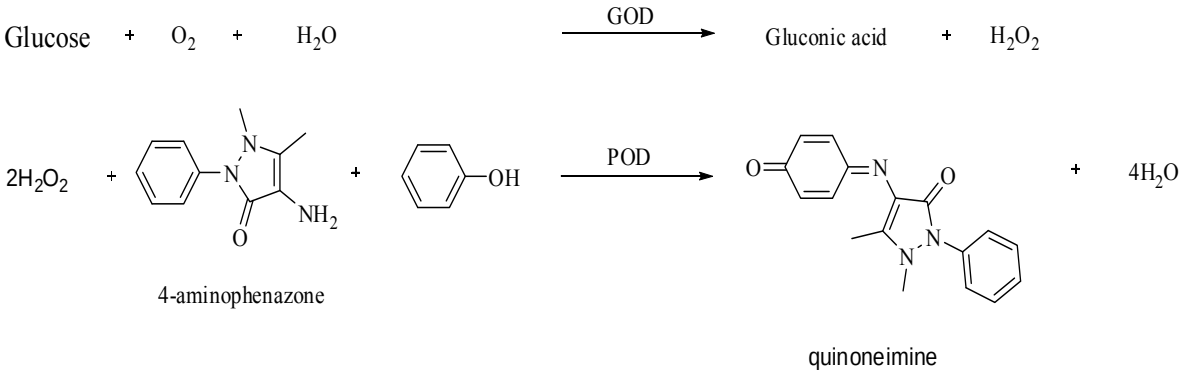


p-nitrophenyl-α-D-glucopyranoside *p*-nitrophenol glucose

Figure 2.7 The hydrolysis of *p*NPG α-glucosidase from baker's yeast

2.5.4.3 Rat intestinal α-glucosidase inhibitory activity

Rat intestinal α-glucosidase inhibitory activity was determined according to the method of Adisakwattana *et al*. with slight modification. The crude enzyme solution prepared from rat intestinal acetone powder was used as a source of maltase and sucrase. Rat intestinal acetone powder (1 g) was homogenized in 30 mL of 0.9%NaCl solution. After centrifugation (12,000*g* × 30 min), the aliquot was subjected to assay. A 10 μL of synthesized compounds (1 mg/mL in DMSO) was added with 30 μL of the 0.1 M phosphate buffer (pH 6.9), 20 μL of the substrate solution (maltose: 10 mM; sucrose: 100 mM) in 0.1 M phosphate buffer, 80 μL of glucose assay kit, and 20 μL of the crude enzyme solution. The reaction mixture was then incubated at 37 ·C for 10 min (for maltose) and 40 min (for sucrose). The percentage inhibition was calculated by $[(A_0-A_1)/A_0] \times 100$, where A_0 is the absorbance without the sample, and A_1 is the absorbance with the sample. The IC_{50} value was determined from a plot of percentage inhibition versus sample concentration. Acarbose® was used as standard control and the experiment was performed in duplicate.



4-aminophenazone quinoneimine

Figure 2.8 The reaction principle of α-glucosidase from rat small intestine

Supporting information

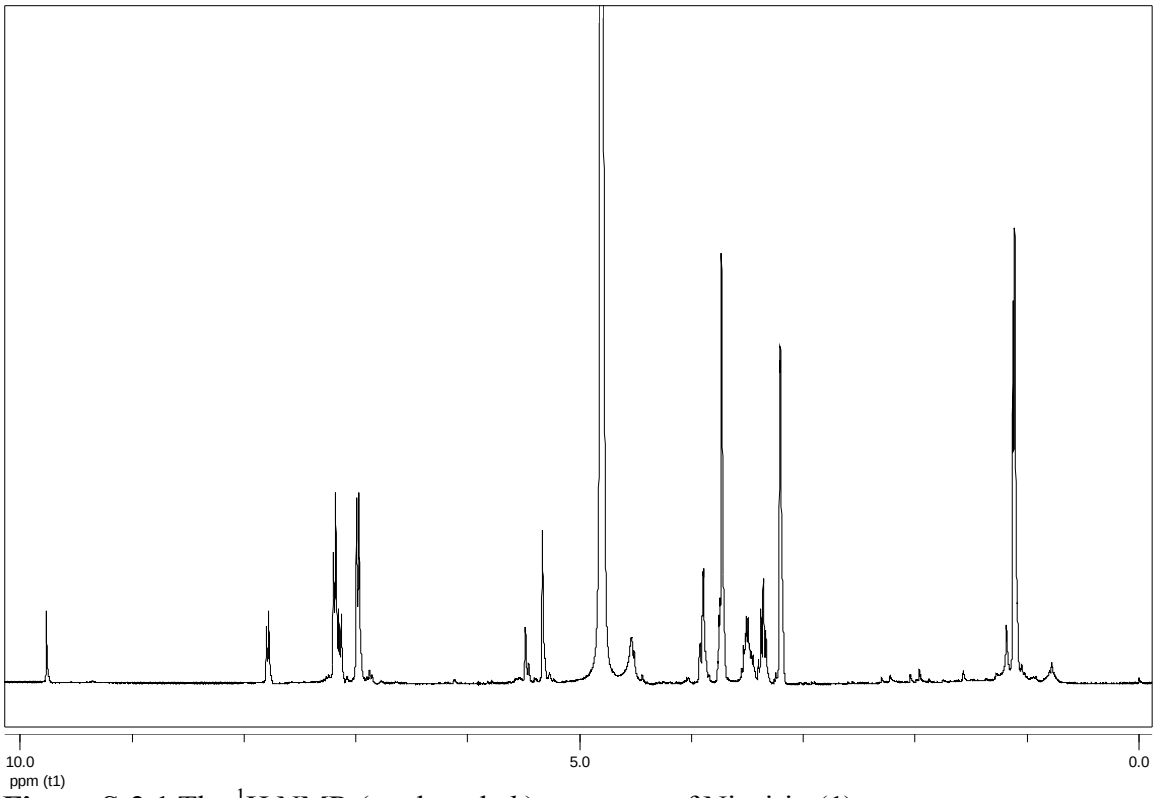


Figure S-2.1 The ^{1}H NMR (methanol-d_4) spectrum of Niazirin (**1**).

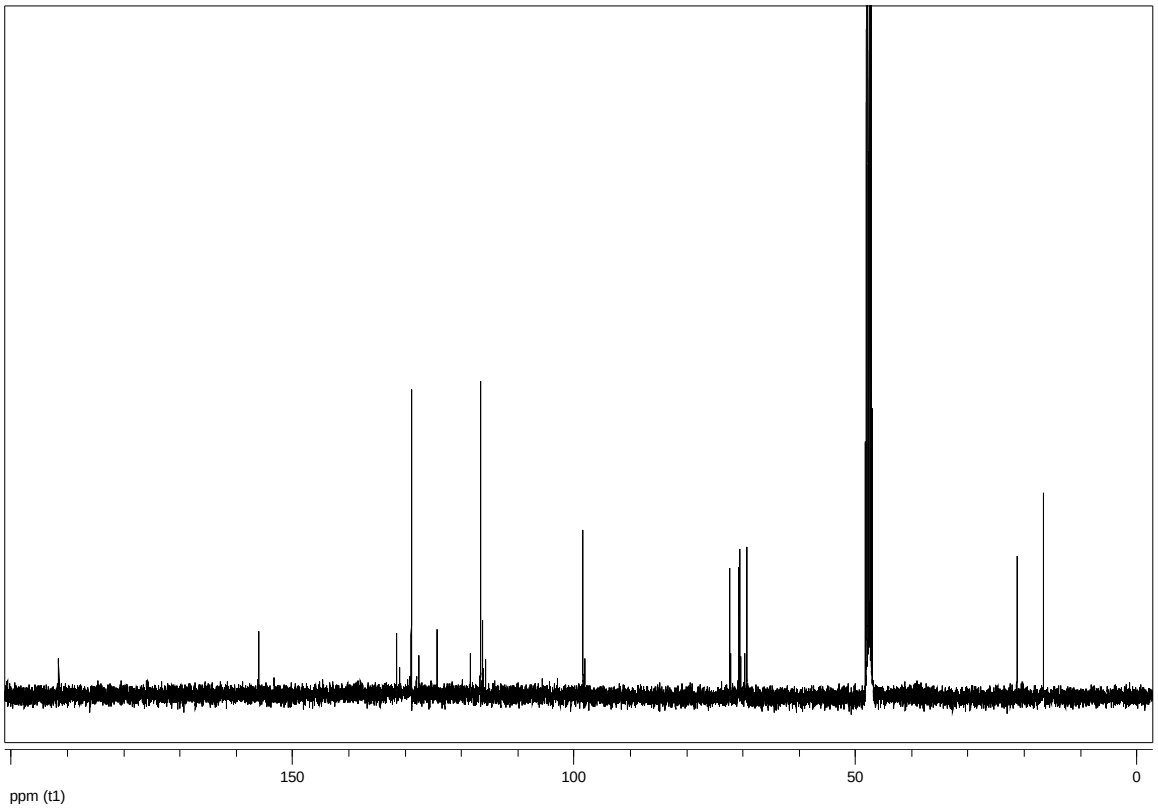


Figure S-2.2 The ^{13}C NMR (methanol-d_4) spectrum of Niazirin (**1**).

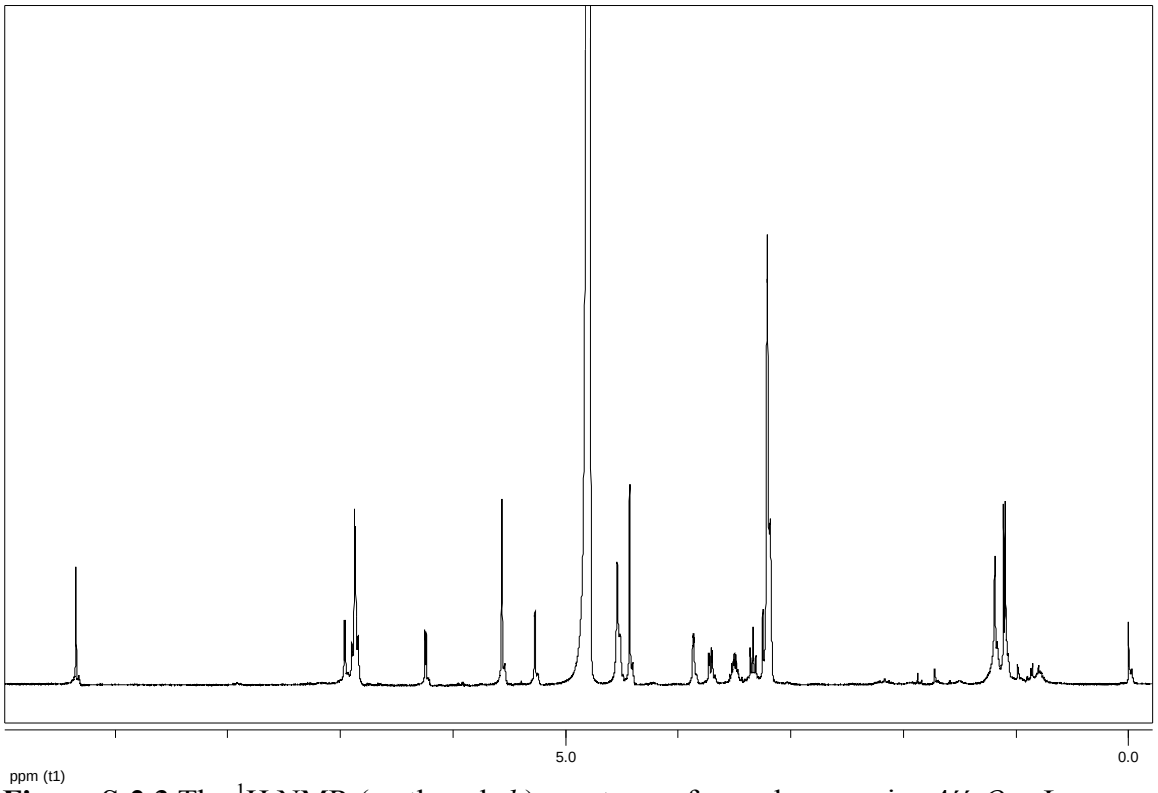


Figure S-2.3 The ^{1}H NMR (methanol-d_4) spectrum of pyrrolemarumine 4′′-*O*-α-L-rhamnopyranoside (**2**).

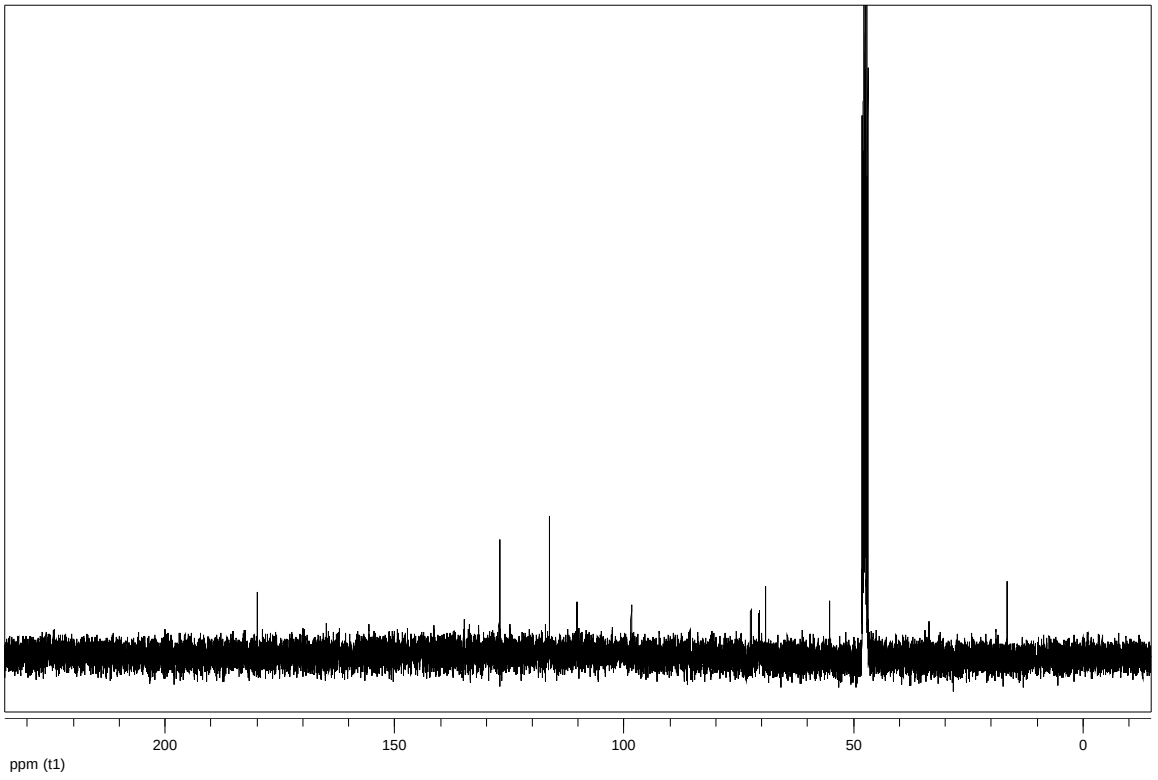


Figure S-2.4 The ^{13}C NMR (methanol-d_4) spectrum of pyrrolemarumine 4′′-*O*-α-L-rhamnopyranoside (**2**).

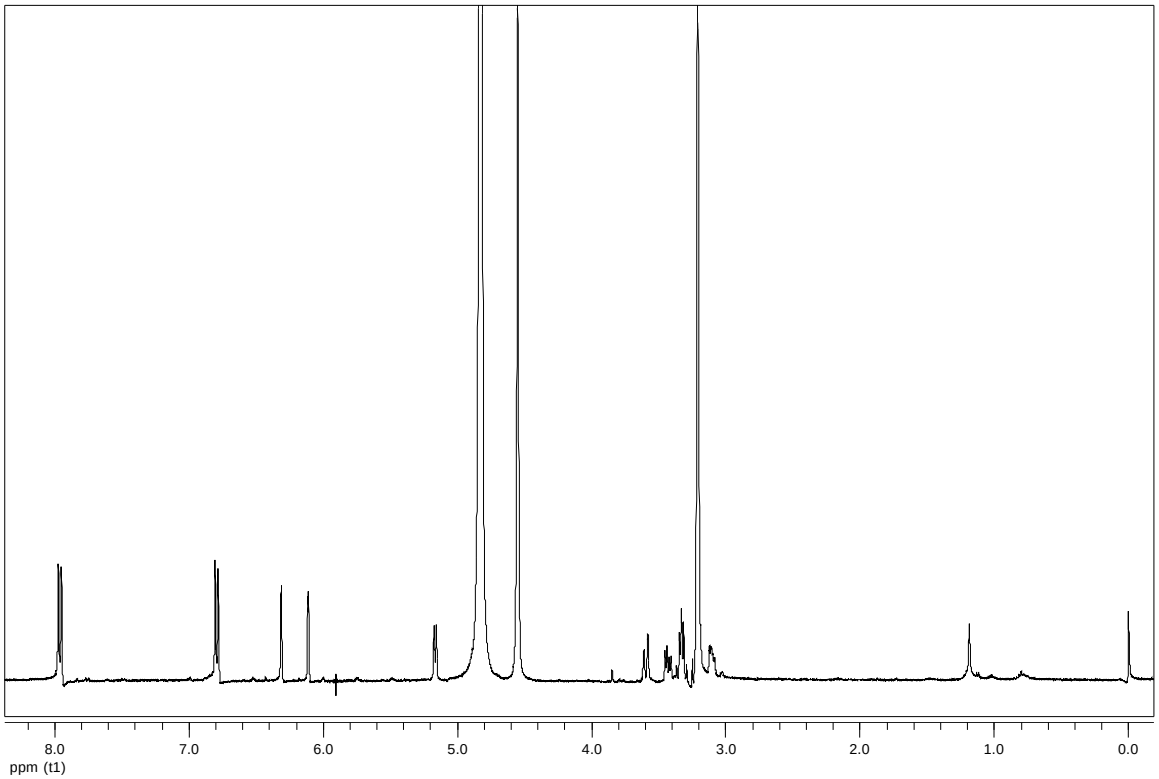


Figure S-2.5 The ^{1}H NMR (methanol-d_4) spectrum of kaempferol-3-*O*-glucopyranoside (**3**).

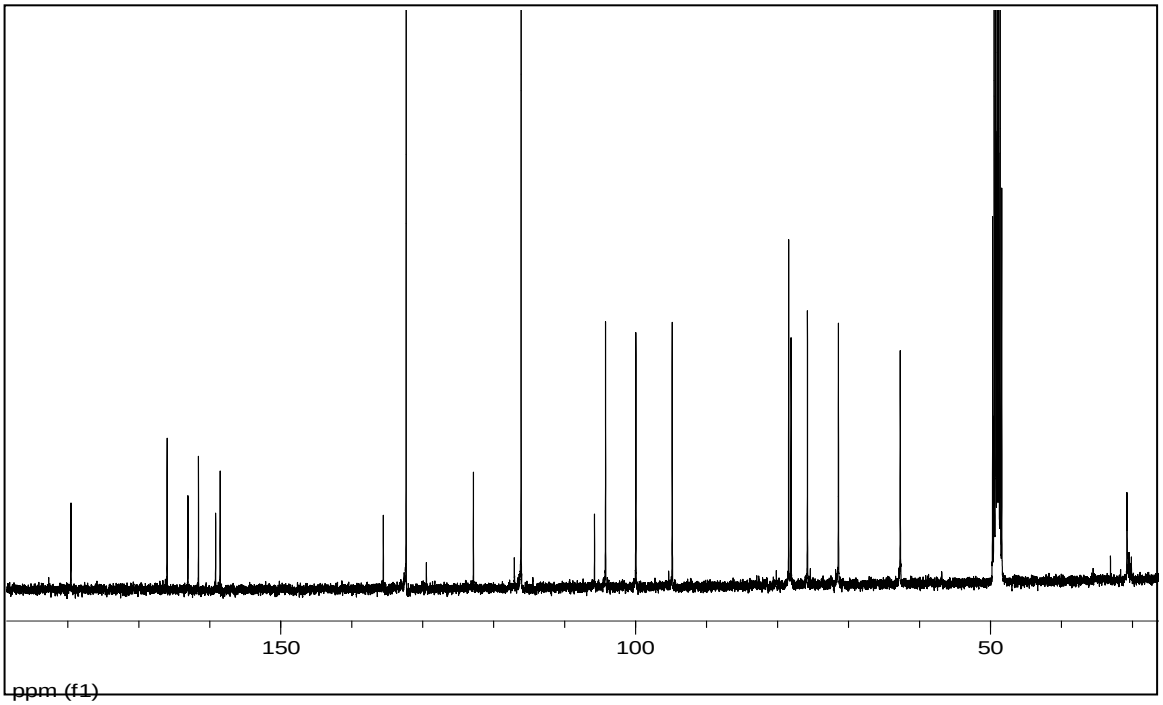


Figure S-2.6 The ^{13}C NMR (methanol-d_4) spectrum of kaempferol-3-*O*-glucopyranoside (**3**).

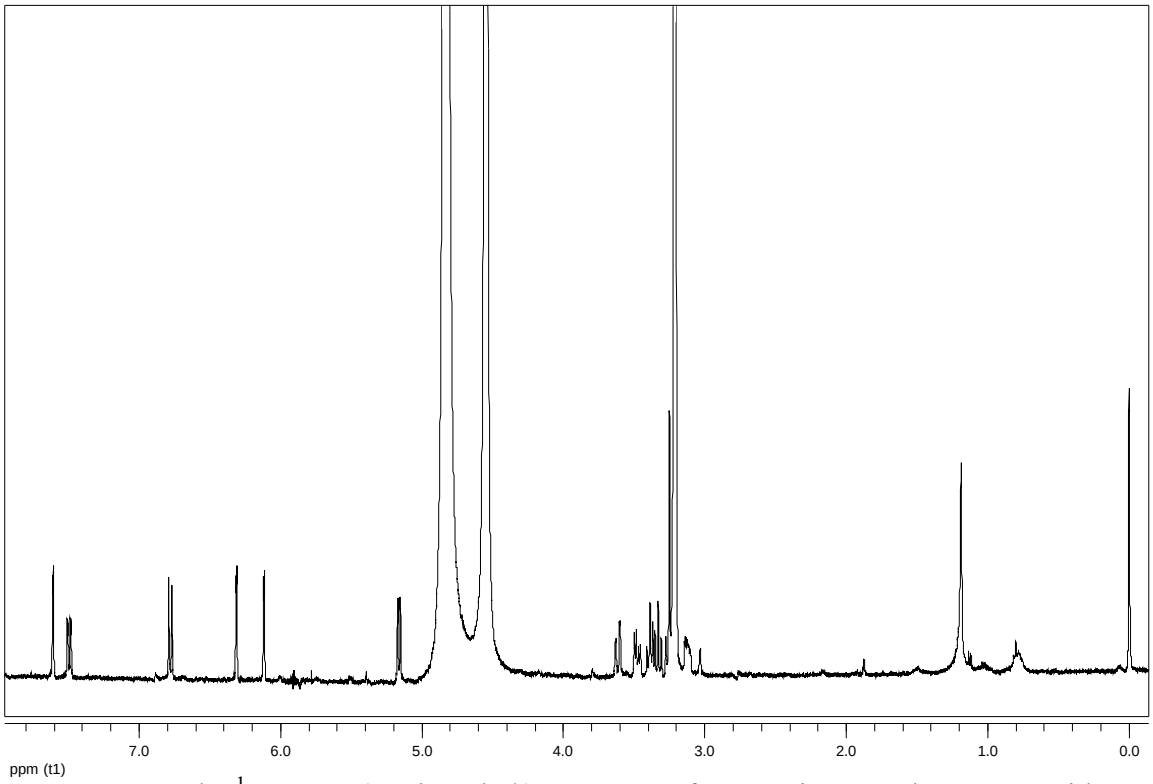


Figure S-2.7 The ^{1}H NMR (methanol-d_4) spectrum of quercetin-3-*O*-glucopyranoside (**4**).

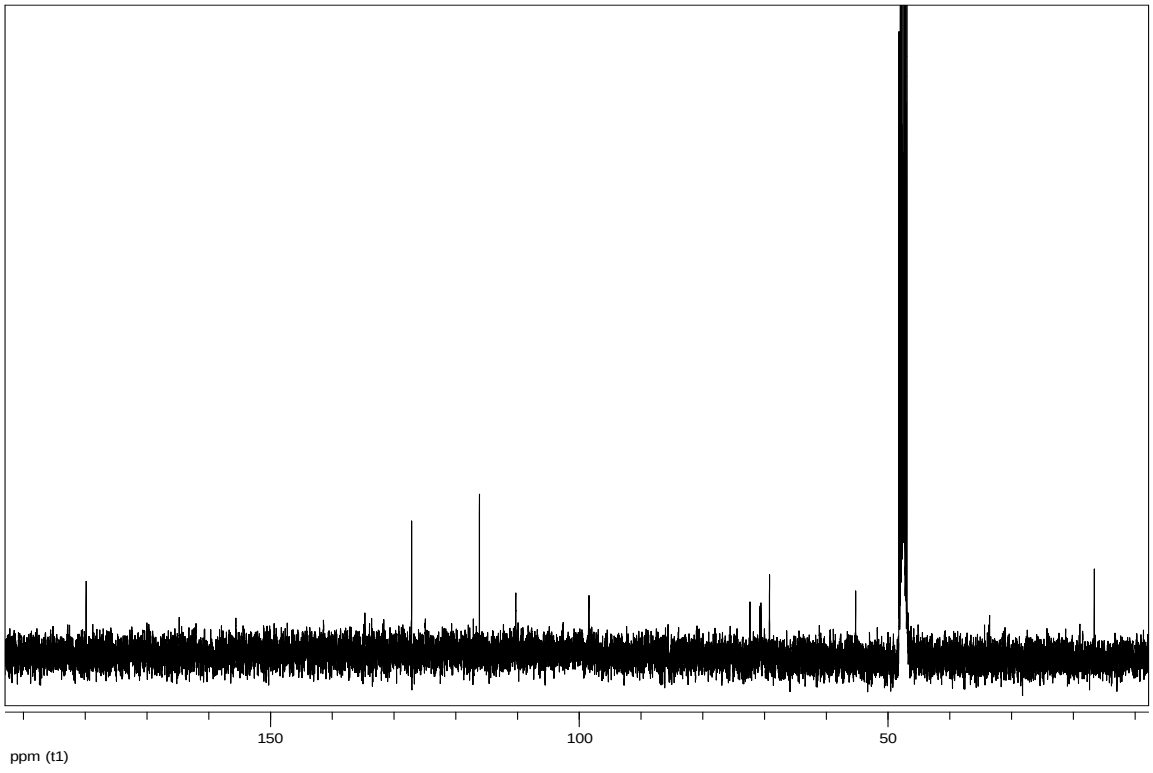


Figure S-2.8 The ^{13}C NMR (methanol-d_4) spectrum of quercetin-3-*O*-glucopyranoside (**4**).

CHAPTER III

α-GLUCOSIDASE INHIBITOR FROM *Sesamum indicum* Seed Pulps

3.1. Introduction

3.1.1 Botanical aspect and distribution of *Sesamum indicum*

Sesamum indicum, commonly known as sesame, belongs to family Pedaliaceae. Many wild relative species occur in Africa, India and tropical regions. Sesame is an annual shrub with white bell-shaped flowers with a hint of blue, red or yellow with branches or without branches. It is grown particularly in tropical regions for the production of seeds, which are rich in oil content. It comes in a variety of colors, cream-white to charcoal-black. It has ultimate economical importance. Sesame grows best in sandy well drained soil with hot weather and moderate rainfall (Chakraborthy *et al*., 2008).

Figure 3.1 *Sesamum indicum*

3.1.2 Nutrition, phytochemical and pharmacological investigation of *Sesamum indicum*

For thousands of years, sesame seeds have been a source of oil and food. Sesame oil is one of the most stable vegetable oils with long shelf life because of the high level of natural antioxidants. Sesame oil, highly containing oleic and linoleic acids (47 and 39 %), is used in cooking as margarine and salad oils (Maozzami *et al*.,

2006). Moreover, it highly contains physiologically active furofuran lignans, especially sesamin and sesamolin (Fig. 3.2), which are responsible for the unique properties of seed oil (Maozzami *et al*., 2007).

In addition to seed oil, defatted seeds obtained after oil production are used as protein source (Kang *et al*., 1999) in animal feed. Defatted seeds mainly contain lignan glycosides such as pinoresinol glycosides and sesaminol glycosides (Fig 3.3) (Maozzami *et al*., 2007).

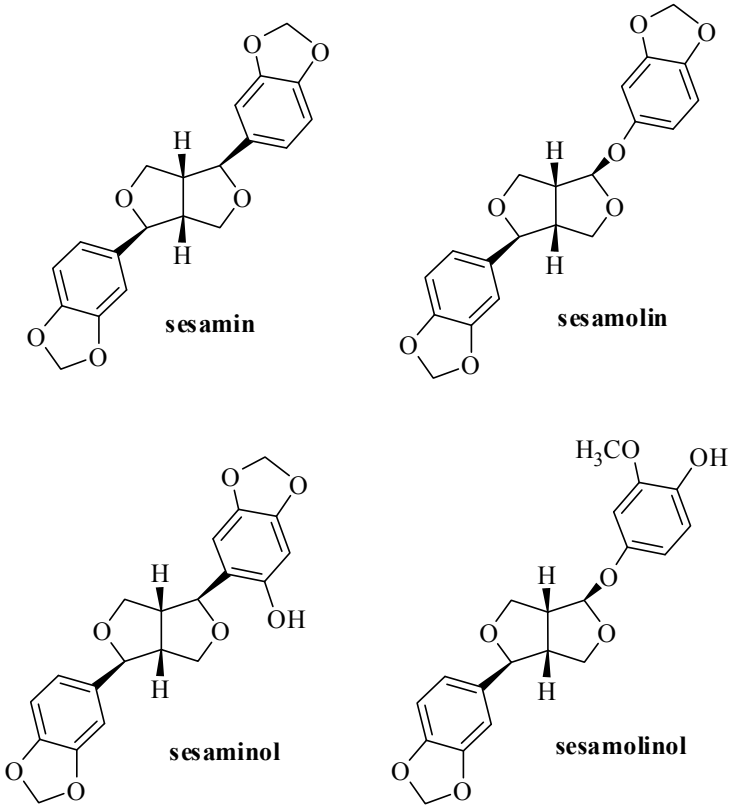
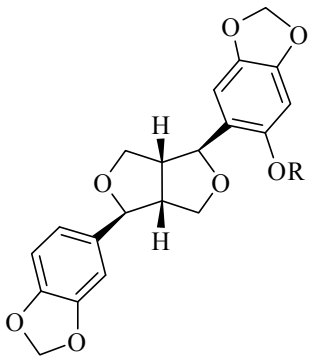
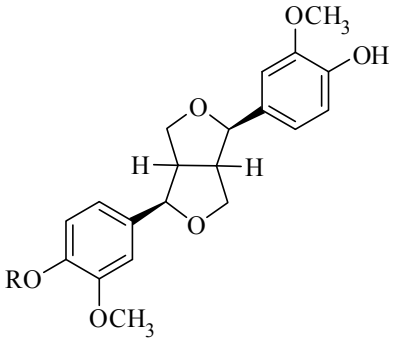


Figure 3.2 Major lignans in *Sesamum indicum* seeds



Sesaminol monoglucoside	R = Glc
Sesaminol diglucoside	R = Glc-Glc
Sesaminol triglucoside	R = Glc-Glc-Glc



Pinoresinol monoglucoside	R = Glc
Pinoresinol diglucoside	R = Glc-Glc
Pinoresinol triglucoside	R = Glc-Glc-Glc

Figure 3.3 Lignan glucosides in defatted sesame seeds.

Although there are many pharmacological reports of sesame oil (Ahmad *et al*., 2006; Fukuda *et al*., 1996; Kaur and Saini *et al*., 2000), those of defatted seeds were limited. However, this research concentrated on defatted seed, was reported to have neuroprotection (Jamarkattel-Pandit *et al*., 2010), reduce brain oedema (Lee *et al*., 2012) decreases oxidative stress (Kang *et al*., 1999), antioxidative (Lee *et al*., 2005) and hypoglycemic effects.

Hypoglycemic effect of defatted sesame seeds in genetically diabetic KK-A^y mice was first discovered by Takeuchi and coworkers (Takeuchi *et al*., 2001).

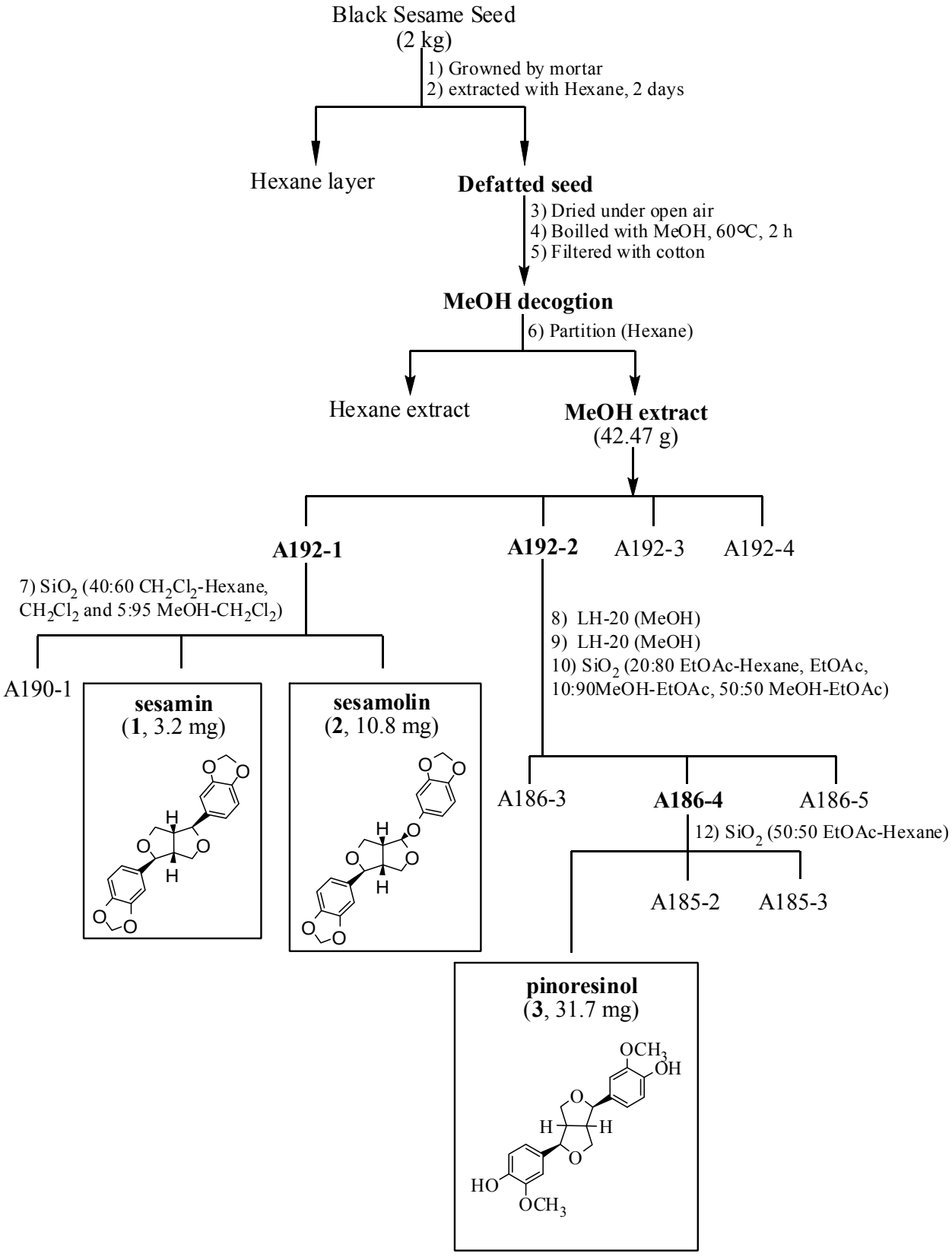
The experiment minces treated with hot water extract for four weeks showed 27% reduction in urinary glucose, compared with controls. Further investigation indicated that hot water extract of sesame seed significantly suppressed plasma glucose of mice fed with maltose solution within 2 h. However, the plasma glucose reduction has no connection with insulin-releasing stimulation. Therefore, we hypothesize that hypoglycemic effect of sesame seeds is likely to be associated with α-glucosidase inhibition. In addition to animal model, hypoglycemic effect of sesame seeds was also detected in type 2 diabetic women (Figueiredo *et al*., 2008).

From aforementioned reports, we concluded that sesame defatted seeds possess hypoglycemic effect possibly through α-glucosidase inhibition. Therefore, this research is aimed at identifying active components responsible for inhibiting α-glucosidase function.

3.2 Isolation

The seeds of *Sesamum indicum* (2 kg) were ground and extracted with hexane for 2 days to obtain hexane layer and defatted seed. The seed residues were dried, boiled with MeOH at 60 °C for 2 h to obtain MeOH decoction, which was in turn partitioned with hexane to ensure the removal of seed oil. The MeOH extract was evaporated to dryness and separated through vacuum column chromatography using stepwise CH_2Cl_2, MeOH-CH_2Cl_2, respectively, yielding four major fractions (A192-1 to A192-4). Fraction A192-1 was separated through silica gel CC with 40:60 CH_2Cl_2-hexane, CH_2Cl_2 and 5:95 MeOH-CH_2Cl_2 to afford two lignans named sesamin (**1**) and sesamolin (**2**). Fraction A192-2 was purified by sephadex LH-20 column with MeOH followed by silica gel CC with mixture of MeOH-EtOAc to afford pinoresinol (**3**).

To further investigate major components in hot water extract and methanol extract are whether identical, we followed Takeuchi and coworkers protocol with slight modification. Defatted seeds were boiled with water instead of methanol. The water decoction was separated by Dianion HP-20 eluted by water and methanol. The combined fractions eluted with methanol were purified by VCC (MeOH-CH_2Cl_2) to afford four major fractions (AW2-1 to AW2-4). However, only pinoresinol was detected, in fraction AW2-2.



Scheme 3.1 Isolation of *Sesamum indicum* defatted seeds.

3.3 Structure elucidation of isolated compounds 1-3

Compound **1** was obtained as a canary yellow amorphous. The structure was deduced by the results from ^{1}H, ^{13}C NMR spectroscopic methods. The ^{1}H NMR spectral displayed doublet methane proton signal at δ_H 3.05 (J = 1.2 Hz, H-5 and H-2, respectively). The methylene proton, showed that doublet of doublet axial proton signal at δ_H 3.87 (J = 6 Hz) and doublet of doublet equatorial proton signal at δ_H 4.23 (J = 2 Hz) were assigned as boyh pf H-4 and H-8. The doublet benzylic proton at δ_H 4.71 (J = 3.6 Hz) could be ascribed to H-2 and H-6. The singlet proton at δ_H 5.95 integrated as 4 protons were assigned to two methylenedioxy group. whereas two ABX system at δ_H 6.79 (dd, J = 2 Hz, H-5', H-6' and H-5" and H-6") and δ_H 7.26 (s) were assigned as H-2' and H-2". The ^{13}C NMR spectral was confirmed. Therefore, the structure of compound **1** as the same structure of sesamin (Khaleel *et al.,* 2007).

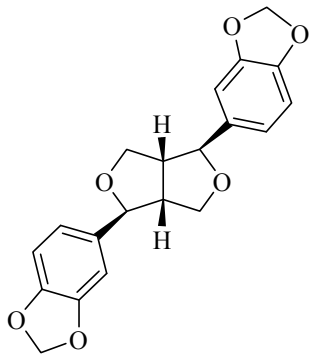


Figure 3.4 sesamin (**1**)

Compound **2** was obtained as a canary yellow amorphous. The structure was deduced by the results from ^{1}H, ^{13}C NMR spectroscopic methods. The ^{1}H NMR spectral showed two quatet methine protons at δ_H 2.94 and 3.31 (each J = 7.6 Hz, H-5 and H-1, respectively). Two pair of methylene proton signals at δ_H 3.64 (q, J = 1.6 Hz) and 4.42 (m, J = 8.9 Hz) were assigned as H-8, whereas δ_H 3.96 (d, J = 9.2 Hz) and 4.41 (q, J = 3.2 Hz) were assigned as H-4. Two benzylic proton signals at δ_H 4.41 (m, J = 8.4 Hz) and 5.50 (s) could be ascribed to H-6 and H-2, respectively. In addition, two singlet proton signals at δ_H 5.92 and 5.96 were assigned to the methylenedioxy protons. While, two ABX system at δ_H 6.62-6.81 assigned to H-6", H-2", H-5" and H-5', H-6', H-2', respectively. The ^{13}C NMR spectral was confirmed. Therefore, the structure of compound **2** as the same structure of sesamolin (Khaleel *et al.,* 2007).

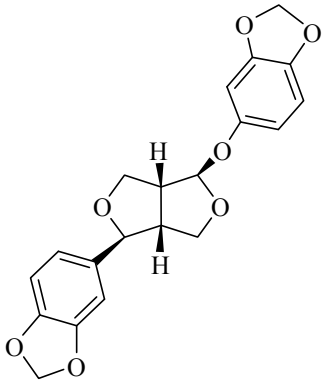


Figure 3.5 sesamolin (**2**)

Compound **3** was obtained as a canary yellow amorphous. The structure was deduced by the results from ^{1}H, ^{13}C NMR spectroscopic methods. The ^{1}H NMR spectral dedicated symmetryly structure. Doublet methane protons at δ_H 3.04 (J = 1.6 Hz, H-1 and H-5). The methylene proton, showed that axial proton at 3.81 and equatorial proton at δ_H 4.24 (each dd, J = 16 Hz) were assigned as both of H-4 and H-8. The doublet benzylic proton signals at δ_H 4.67 (J = 4 Hz) could be ascribed to H-2 and H-6. In addition to aromatic ring, were showed proton signals at δ_H 6.81 (s), 6.75 (dd, J = 8 Hz) and 6.83 (d, J = 1.6 Hz) were assigned as H-2', H-5' and H-6', respectively. Whereas, singlet signals at δ_H 5.54 and 3.90 were characterized to hydroxyl proton and methoxyl proton, respectively. The ^{13}C NMR spectral was confirmed. Therefore, the structure of compound **3** as the same structure of pinoresinol (Miyazawa *et al.,* 1992).

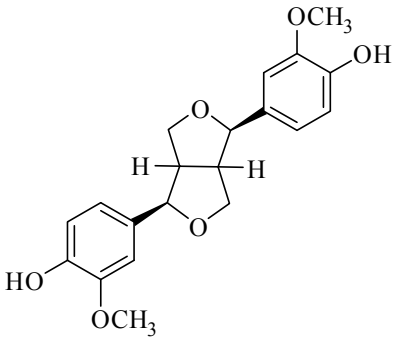


Figure 3.6 pinoresinol (**3**)

3.4 α-Glucosidase inhibitory activity of the isolated compounds

The α-glucosidase inhibitory activity of compounds **1-3** isolated from *Sesamum indicum* defatted seeds was evaluated by colorimetric method and the results are shown in Table 3.1

Table 3.1 α-glucosidase inhibitory activity of isolated compounds from *Sesamum indicum* defatted seeds.

compounds	IC_{50} (mM) [a]		
	α-glucosidase		
	Baker's yeast	Rat small intestine	
		maltase	sucrase
1	0.45	NI[b]	NI
2	0.20	NI	NI
3	0.49	0.0343	NI
acarbose®	0.22	0.0015	0.0024

[a] The IC_{50}value is defined as the inhibitor concentration to inhibit 50% of enzyme activity.
[b] No inhibition, less than 50% inhibition at 10 mg/ml.

From Table 3.1, lignans **1-3** broadly inhibited α-glucosidase from baker's yeast, whereas pinoresinol (**3**) selectively inhibited maltase from rat intestine (IC_{50} 0.0343 mM). Since, α-glucosidase in human brush border and rat are categorized into the same group. Therefore, we concluded that compound **3** was an active component responsible for hyperglycemic effect.

To gain insight into inhibition mechanism of pinoresinol, kinetic study was carried out. Kinetic analysis of inhibitors was calculated by using Lineweaver-Burk plot. The Lineweaver-Burk plot of different concentration of pinoresinol produced series of straight lines, all of which intersected in the second quadrant. The analysis showed that K_m increased with decreased V_{max} values (Table 3.2). Therefore Figure 3.8, indicating that pinoresinol possesses mixed-type inhibition.

The possible binding mode of pinoresinol was presumed that one inhibitor can bind either to active site of free enzyme (**E**) in competitive manner or to the enzyme-substrate complex (**ES**) in noncompetitive manner.

To gain insight into binding affinity of inhibitor (**I**) to free enzyme and ES complex, dissociation constants K_i and K_i' were determined.

Dixon plot (Fig 3.8) showed the dissociation constant of EI complex with K_i value of 0.28 mM, whereas secondary replot (Fig 3.9) showed the dissociation constant of ESI complex with K_i' value of 1.34 mM.

Apparently, K_i value of pinoresinol was approximately 4 times less than K_i' value, indicating that binding of pinoresinol to the ES complex was weaker than that of pinoresinol to free enzyme. In addition, the data implied that pinoresinol is dominant in competitive inhibition.

Table 3.2 Inhibition mechanism.

Type of inhibition	K_m	V_{max}	Intersection
Competitive	increase	unchanged	Y axis, Y>0
Non-competitive	unchange	decrease	X axis, X<0
Uncompetitive	decrease	decrease	no intersection
Mixed	increase	decrease	second quadrant

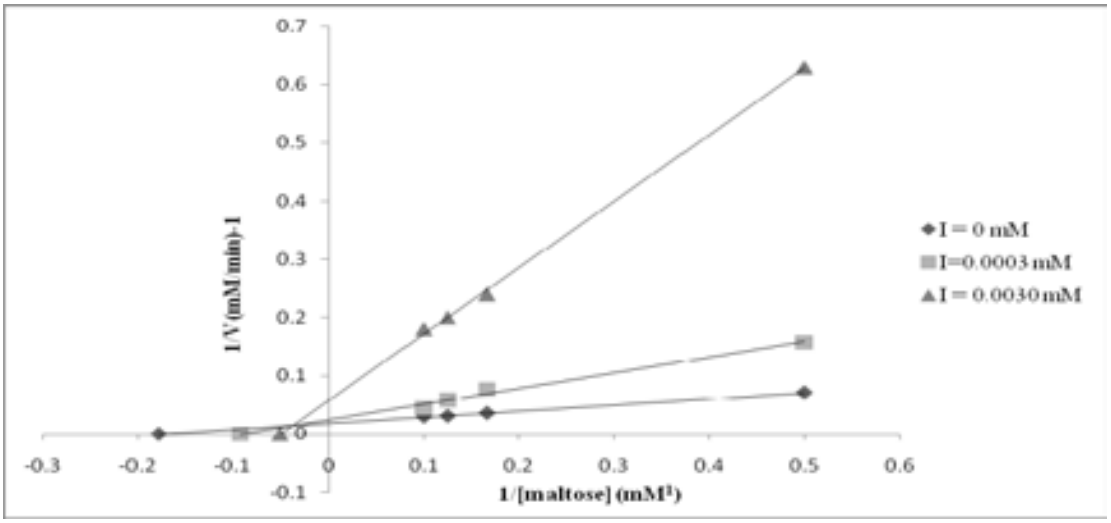
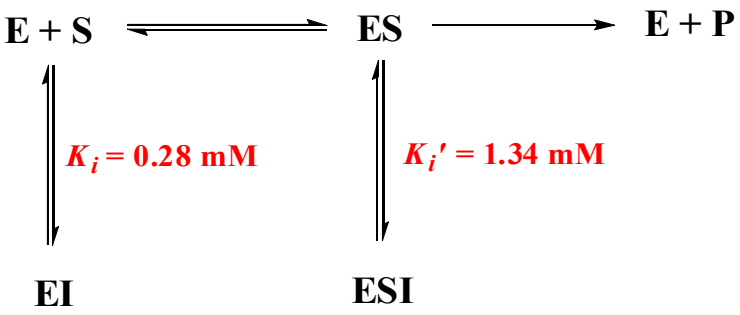


Figure 3.7 The data were presented in a Lineweaver-Burk plot, 1/*v* against 1/[*S*] of pinoresinol.



Scheme 3.2 Inhibition mechanism of pinoresinol against maltase

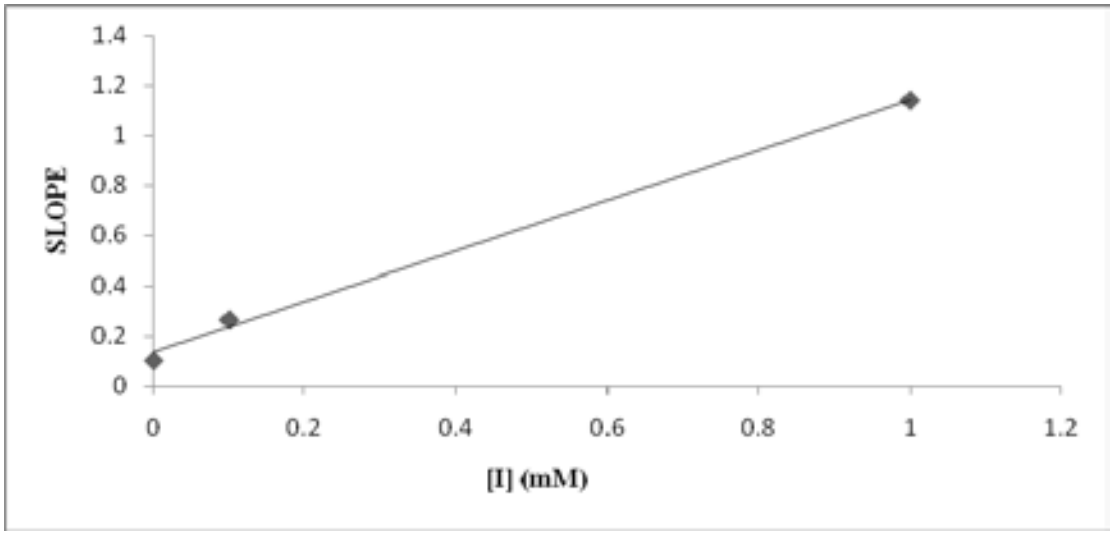


Figure 3.8 Dixon plot of slope vs. [I] from a primary Lineweaver-Burk plot for the determination of K_i.

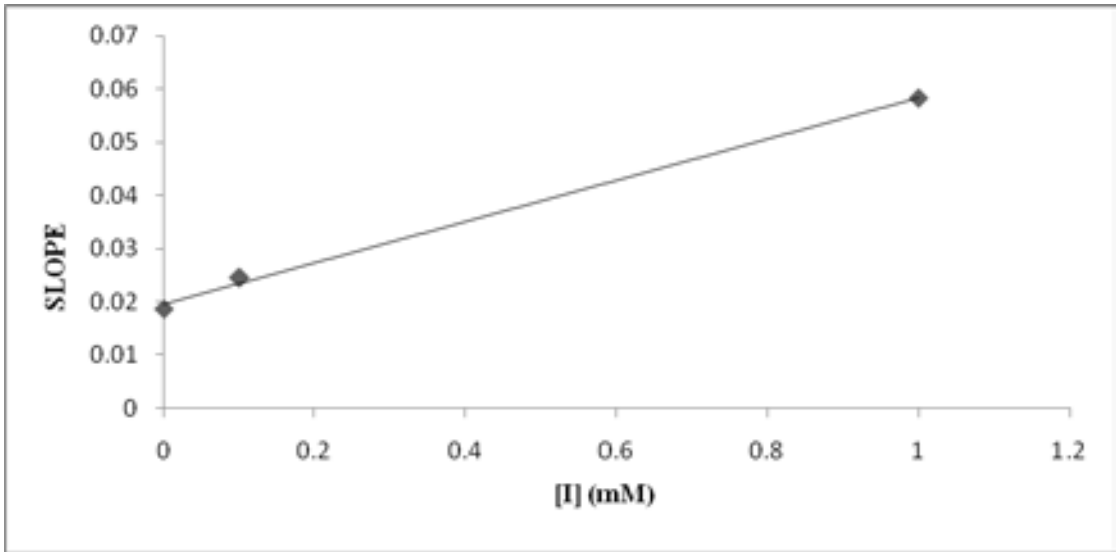


Figure 3.9 Secondary replot plot slope vs. [I] from a primary Lineweaver-Burk plot for the determination of K_i'.

3.5 Experiment section

3.5.1 General experiment procedures

The ^{1}H and ^{13}C NMR spectra (in $CDCl_3$, CD_3OD and acetone-d_6) were determined with a nuclear magnetic resonance spectrometer of Varian model Mercury+ 400. The chemical shift in δ (ppm) was assigned with reference to the signal from the residual protons in deuterated solvents and using TMS as an internal standard in some cases. ESIMS was obtained from Mass Spectrometer Model VG TRIO 2000. Sephadex LH-20 and silica gel 60 Merck cat. No. 7734 and 7729 were used for open column chromatography. Thin layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm thick layer).

3.5.2 Plant material

The seed pulps of *Sesamum indicum* were purchased from Thai Cereals World Co., Ltd (Raithip). Thailand in May 2010

3.5.3 Extraction and isolation

The seeds of *Sesamum indicum* (2 kg) were growned and extracted with Hexane for 2 days to obtain Hexane layer and Defatted seed. The defatted seed was dried, boiled with MeOH at 60 °C for 2 h and filterd with cotton to obtain MeOH decoction. It was partitioned with Hexane for 2 times to obtain Hexane extract and MeOH extract. The MeOH extract was evaporated to dryness and separated through vacuum column chromatography using stepwise CH_2Cl_2, MeOH-CH_2Cl_2 (10:90, 20:80, 30:70 and 40:60), respectively, yielding four major fraction (A192-1 to A192-4). Fraction A192-1 was separated through silica gel CC with 40:60 CH_2Cl_2-Hexane, CH_2Cl_2 and 5:95 MeOH-CH_2Cl_2 to afford two lignans named sesamin (**1**, 3.2 mg) and sesaminol (**2**, 10.8 mg). Fraction A192-2 was purified by sephadex LH-20 column with MeOH. Since, was purifired by silica gel CC with 20:80 EtOAc-hexane, EtOAc and MeOH-EtOAc (10:90 and 50:50), respectively, afforded lignin named pinoresinol (**3**, 3.17 mg).

For alternative method, The seeds of *Sesamum indicum* (1 kg) were extracted by soxhlet with Hexane to obtain hexane extract and defatted sesame seeds. The defatted sesame seeds was boiled with H_2O at room temperature for 1 h to obtain Hot

water extract (5 L). This ectract was separated through dianion HP-20 with H_2O and MeOH obtaibed MeOH extract. The MeOH extract was purified by vacuum column chromatography using stepwise followed by procedure I, yielding four major fractions. Subfraction AW2-2 showed lignin named pinoresinol (**3**).

sesamin (1): as Canary Yellow solid;^{1}H NMR (400 MHz, chloroform-d_1) δ 7.26 (1H, s, H-2' and H-2"), 6.79 (4H, q, *J* = 2 Hz, H-5', H-6' and H-5", H-6"), 5.95 (4H, s, H-OMe), 4.71 (2H, d, *J* = 3.6 Hz, H-2 and H-6), 4.23 (2H, dd, *J* = 2 Hz, H-4 and H-8), 3.87 (2H, dd, *J* = 6 Hz, H-4 and H-8), 3.05 (2H, d, *J* = 1.2 Hz, H-1 and H-5). ^{13}C NMR (400 MHz, chloroform-d_1) δ 147.9 (C-3' and C-3"), 147.1 (C-4' and C-4"), 135.0 (C-1' and C-1"), 119.4 (C-6' and C-6"), 108.6 (C-5' and C-5"), 106.5 (C-2' and C-2"), 101.1 (C-7' and C-7"), 85.8 (C-2 and C-6), 71.7 (C-4 and C-8), 54.3 (C-1 and C-5).

sesamolin (2): as Canary Yellow solid;^{1}H NMR (400 MHz, chloroform-d_1) δ 7.26 (2H, s, H-2'), 6.81 (4H, m, H-5' and H-6'), 6.72 (1H, s, H-5"), 6.70 (1H, s, H-2"), 6.62 (2H, d, *J* = 2 Hz, H-6"), 5.96 (4H, s, -7"), 5.92 (4H, s, H-7), 5.50 (2H, s, H-2), 4.42 (4H, m, H-6 and H-8), 4.12 (2H, q, *J* = 3.2 Hz, H-4), 3.96 (2H, d, *J* = 9.2 Hz, H-4), 3.64 (2H, q, *J* = 1.6 Hz, H-8), 3.31 (2H, q, *J* = 7.6 Hz, H-1), 2.94 (2H, m, H-5). ^{13}C NMR (400 MHz, chloroform-d_1) δ 151.8 (C-1"), 148.1 (C-3"), 148.0 (C-3'), 147.1 (C-4'), 142.7 (C-4"), 134.4 (C-1'), 119.7 (C-6'), 109.0 (C-5"), 108.2 (C-5'), 106.9 (C-6"), 106.5 (C-2'), 101.2 (C-2), 101.1 (C-2"), 100.1 (C-7' and C-7"), 87.0 (C-6), 71.3 (C-4), 69.8 (C-8), 53.2 (C-5), 52.7 (C-1).

pinoresinol (3): as Canary Yellow solid;^{1}H NMR (400 MHz, chloroform-d_1) δ 6.83 (2H, d, *J* = 1.6 Hz, H-6' and H-6"), 6.81 (1H, s, H-2' and 2"), 6.75 (2H, dd, *J* = 8 Hz, H-5' and H-5"), 5.54 (s, H-OH), 4.67 (2H, d, J = 4 Hz, H-2 and H-6), 4.24 (2H, m, H-4 and H-8), 3.90 (1H, s, H-OMe), 3.80 (3H, dd, *J* = 16 Hz, H-4 and H-8), 3.04 (2H, d, *J* = 1.6 Hz, H-1 and H-5). ^{13}C NMR (400 MHz, chloroform-d_1) δ 146.7 (C-3' and C-3"), 145.3 (C-4' and C-4"), 132.9 (C-1' and C-1"), 119.0 (C-6' and C-6"), 114.3 (C-5' and C-5"), 108.7 (C-2' and C-2"), 85.9 (C-2 and C-6), 71.7 (C-4 and C-8), 56.0 (C-OMe), 54.2 (C-1 and C-5).

3.5.4 α-Glucosidase inhibitory assay

α-Glucosidase inhibitory assay was performed the same as previously described in Chapter 2.

3.5.5 Measurement of kinetic constant

Kinetic analysis of maltase was determined using the methods described by Adisakwattana *et al*., Kandra *et al*. and Lineweaver-Burk *et al*., with slight modification. Briefly, the active compounds and enzyme and active compounds were incubated with increasing concentration of maltose (2-20 mM). A Lineweaver-Burk plot of substrate concentration (1/[S]) on horizontal axis and velocity (1/[*V*]) on vertical axis was constructed. K_M and V_{max} were calculated using the following expression.

Which is; $V_{max} = 1/C$ where; C = Y intercept

$$K_M = \text{slope} \times V_{max}$$

For calculation of K_i and K_i' values, slopes from a Lineweaver-Burk plot were replotted vs. [I] which gave the secondary plot. The general equation for the kinetic analysis is

$$V_0 = \frac{V_{max}[S]}{K_m(1+\frac{1}{K_i}[I]) + [S](1+\frac{1}{K_i'}[I])}$$

Where V_0 is the initial velocity of the inhibitor, V_{max} is the limiting velocity. [S] and [I] are the final concentration of substrate and inhibitor, respectively. K_i and K_i' are the dissociation constants of EI and ESI, respectively.

Supporting information

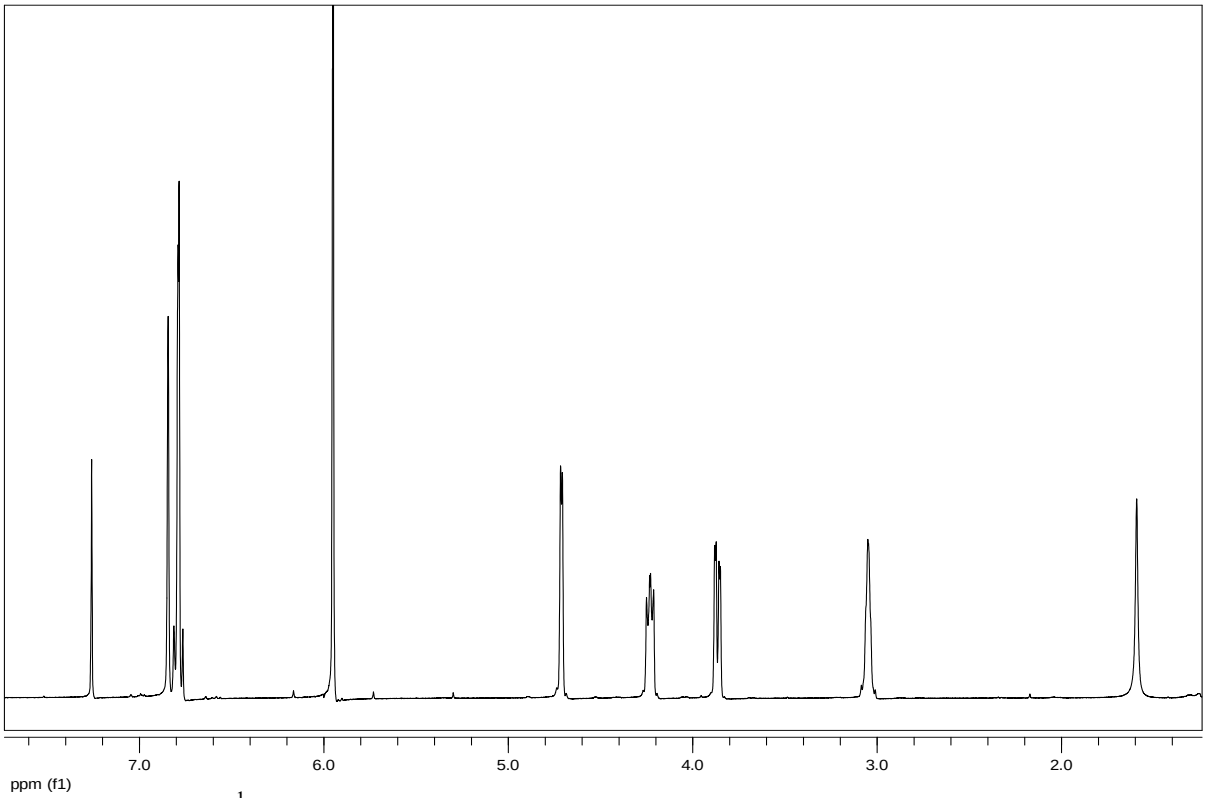


Figure S-3.1 The ^{1}H NMR (chloroform-d_1) spectrum of sesamin (**1**).

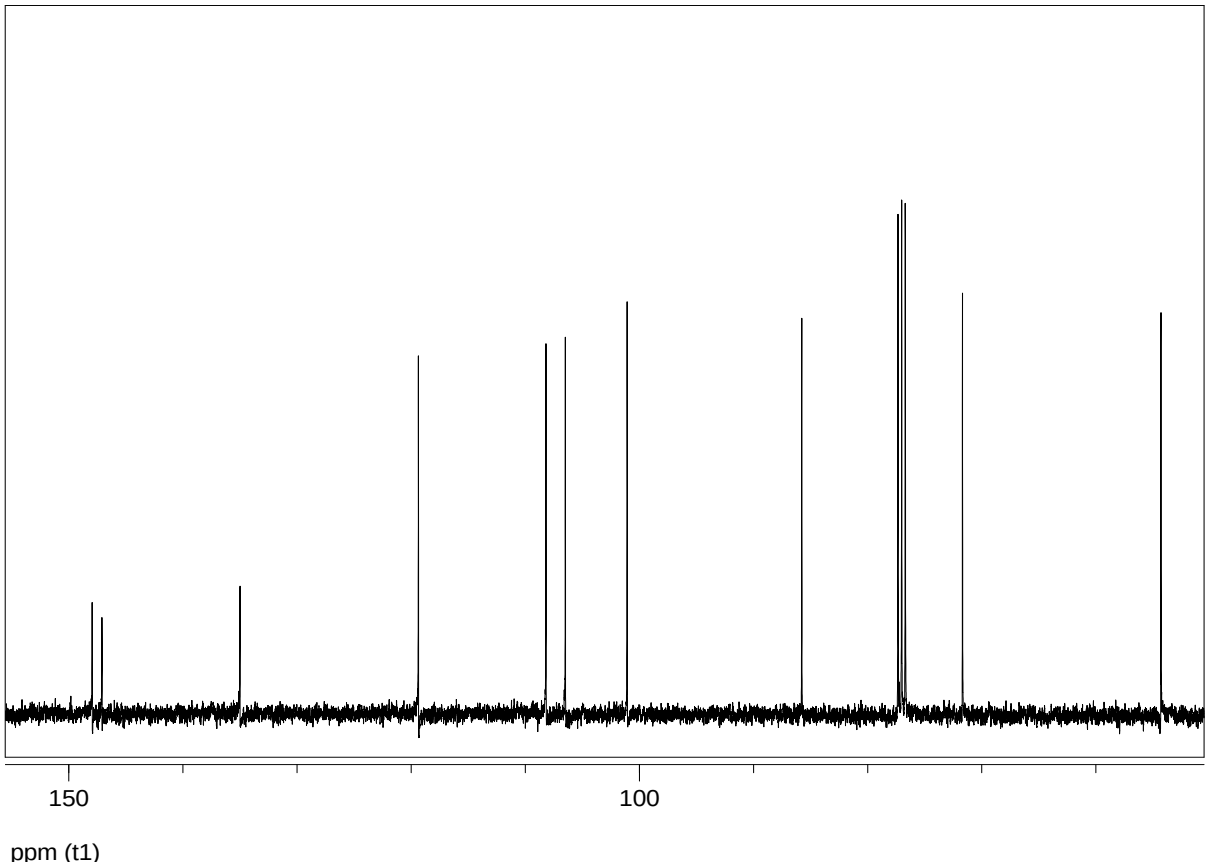


Figure S-3.2 The ^{13}C NMR (chloroform-d_1) spectrum of sesamin (**1**).

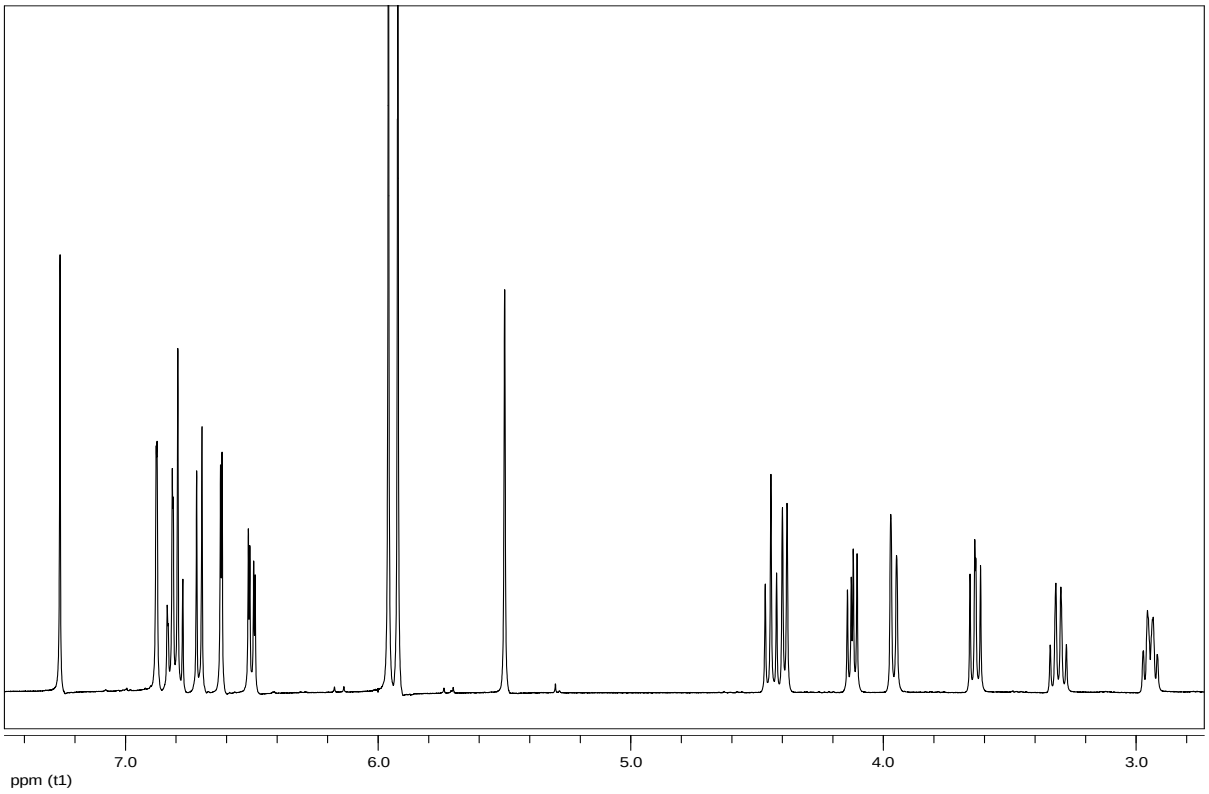


Figure S-3.3 The ^{1}H NMR (chloroform-d_1) spectrum of sesamolin (**2**).

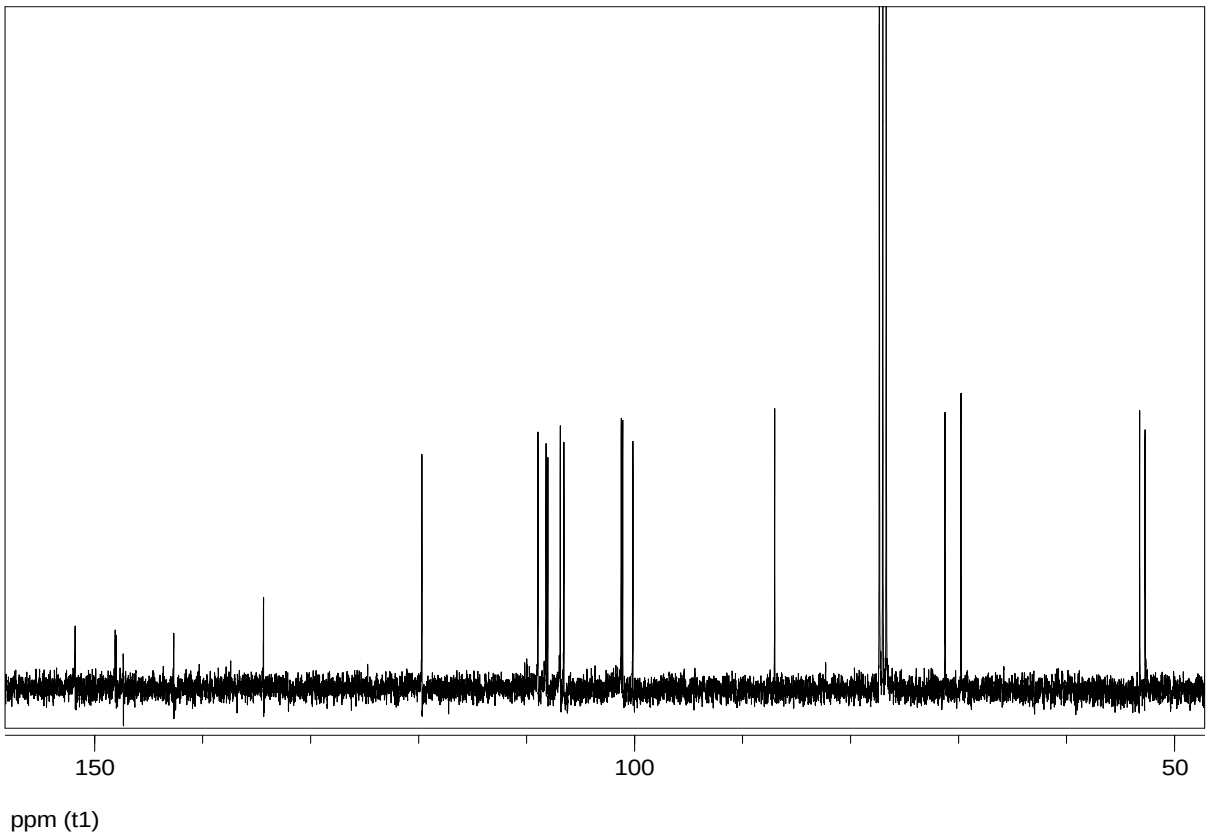


Figure S-3.4 The ^{13}C NMR (chloroform-d_1) spectrum of sesamolin (**2**).

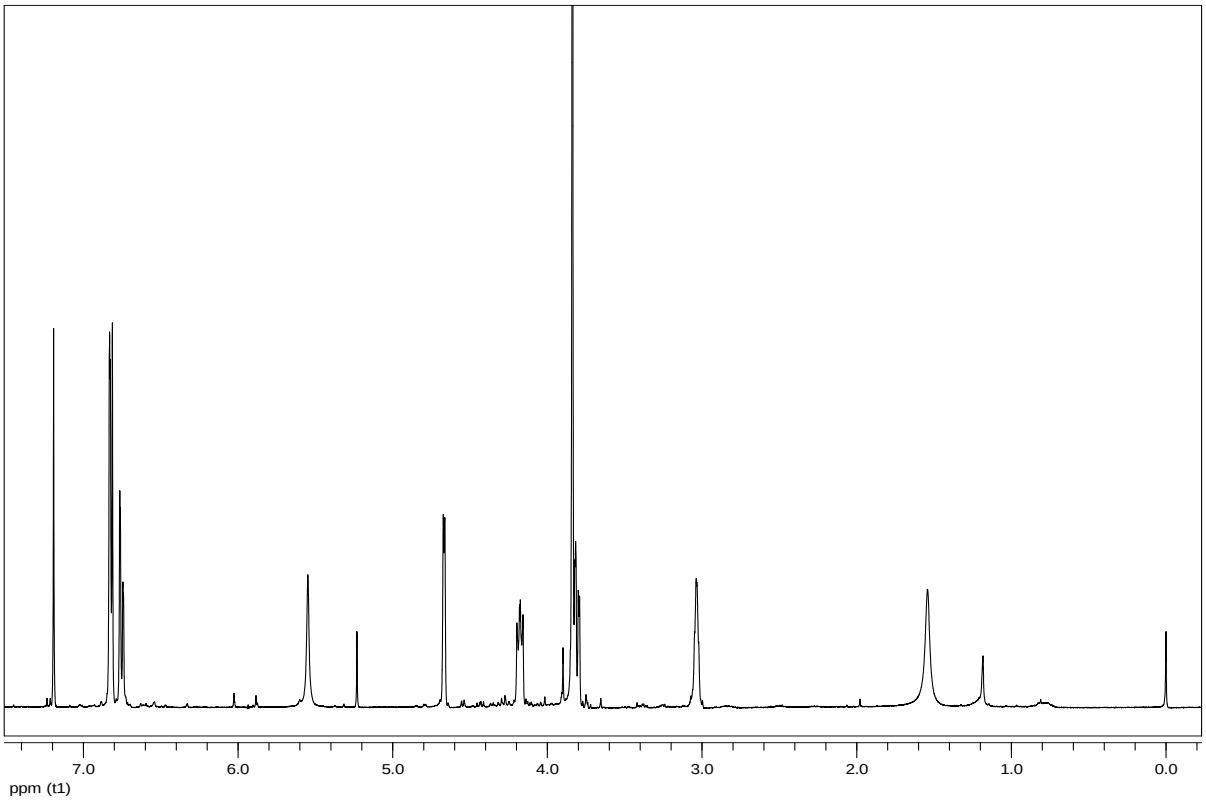


Figure S-3.5 The ^{1}H NMR (chloroform-d_1) spectrum of pinoresinol (**3**).

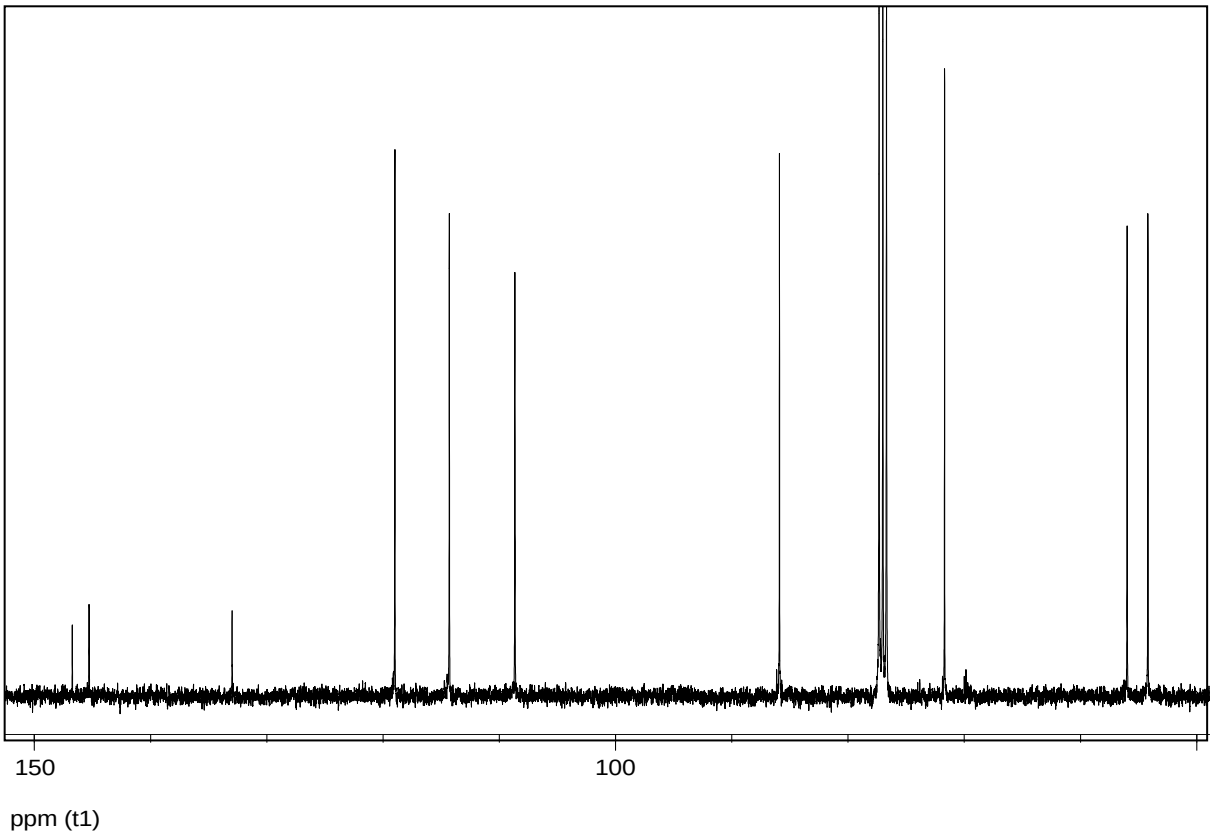
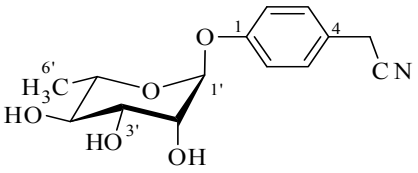


Figure S-3.6 The ^{13}C NMR (chloroform-d_1) spectrum of pinoresinol (**3**).

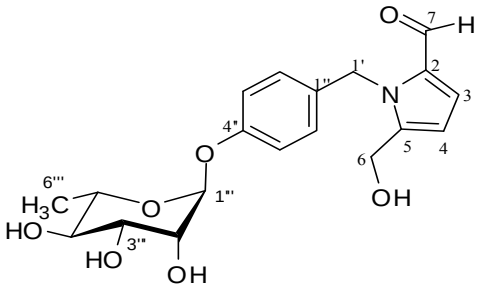
CHAPTER IV

CONCLUSION

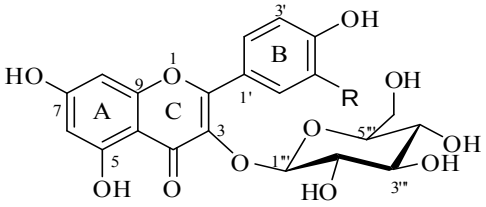
The α-glucosidase inhibitors from *Moringa oleifera* leaves and *Sesamum indicum* defatted seeds were identified. Two phenolic rhamnosides named niazirin and pyrrolemarumine 4"-*O*-α-L-rhamnopyranoside and two flavonoids glycosides named kaempferol-3-*O*-*β*-glucopyranoside and quercetin-3-*O*-*β*-glucopyranoside were isolated from methanol extract of *M.oleifera* leaves. Phenolic rhamnosides exhibited inhibitory effect against α-glucosidase from baker's yeast whereas two flavonoids glycosides showed inhibitory activity against α-glucosidases from rat intestine. On the other hand, three lignans named sesamin , sesamolin and pinoresinol were isolated from the methanol extract of *S.indicum* defatted seeds. They broadly inhibited α-glucosidase from baker's yeast, whereas pinoresinol selectively inhibited maltase from rat intestine. In addition to inhibition machanism of pinoresinol was studied. The kinetic study indicated that this compound possess mix-type inhibition and also dominant in competitive inhibition.



niazirin



pyrrolemarumin 4"-*O*-α-L-rhamnopyranoside



kaempferol-3-*O*-glucopyranoside: R=H
quercetin-3-*O*- glucopyranoside : R=OH

Figure 4.1 The structure of active components from *M.oleifera*

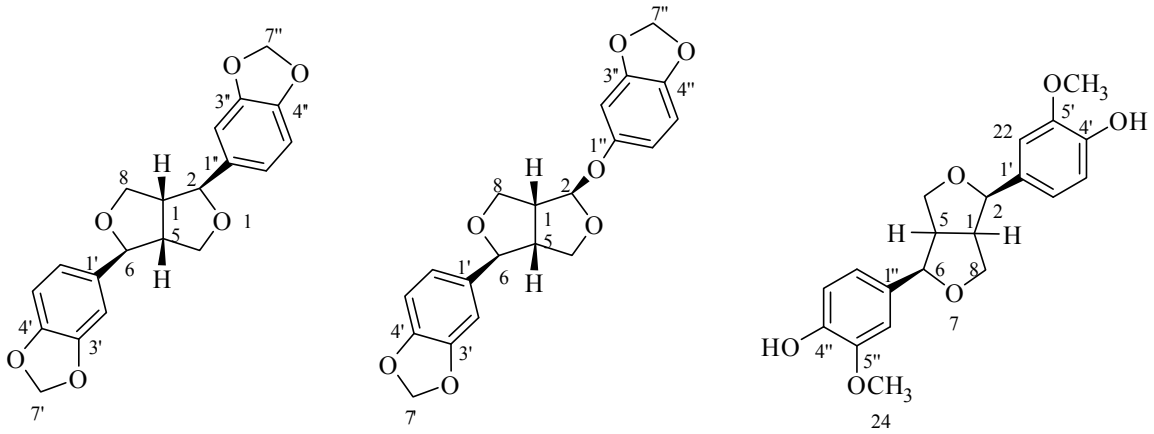


Figure 4.2 The structure of active components from *S.indicum*

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